

Effects of Ethanol on Cartilage Differentiation *In Vitro*

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in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy**

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ABSTRACT

Prenatal alcohol exposure induces diverse developmental anomalies that include skeletal defects of the limbs and face. However the mechanisms underlying its teratogenic actions remain unclear. The objective of my research was to test the hypothesis that ethanol exposure could selectively influence cartilage differentiation in embryonic chondroprogenitor cells. My results demonstrate that 1.0 - 2.0% ethanol treatment significantly elevates both cartilage matrix formation and the expression of cartilage-specific structural genes (type II collagen and aggrecan) in high-density micromass cultures of prechondrogenic mesenchyme isolated from limb buds and facial primordia of stage 23-25 chick embryos. Ethanol treatment stimulated cartilage differentiation even in low-density cultures of limb mesenchyme, and in maxillary and hyoid mesenchyme cultures, which undergo little or no chondrogenesis spontaneously. Ethanol's influence on cartilage differentiation appears to be exerted by the alcohol itself rather than a downstream metabolite since tertiary butanol, which is not metabolized to an aldehyde, was as effective as ethanol in promoting *in vitro* chondrogenesis.

The ability of alcohol to stimulate cartilage differentiation may be restricted to *prechondrogenic* mesenchyme, since ethanol treatment failed to enhance chondrogenesis in cultures of phenotypically-differentiated chondrocytes isolated from the 16-18 day chick embryo sternum. This suggested that ethanol might act at an early point in the chondrocyte differentiation pathway. Consistent with this hypothesis, I found that ethanol exposure rapidly elevated expression of transcripts for three chondrogenic regulatory genes, *Sox-9*, *Ets-2*, and *BMPR-IB*, that are expressed during the prechondrogenic cell aggregation events that precede overt cartilage tissue formation. Although it was recently suggested that alcohol-related birth defects might arise from inhibition of *Msx-2* gene expression, levels of *Msx-2* mRNA were unaffected by ethanol treatment in the limb mesenchyme cultures. My findings indicate that ethanol exposure has the capacity to directly influence embryonic cartilage differentiation and may provide insight into the molecular basis of alcohol's teratogenic effects on skeletal development *in vivo*. (Portions of this work have been reported in Kulyk and Hoffman, 1996, *Exp. Cell Res.* 223:290-300 and in Hoffman and Kulyk, 1999, *Int. J. Dev. Biol.* 43:167-174.)

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TABLE OF CONTENTS

PERMISSION TO USE	i
ABSTRACT	ii
ACKNOWLEDGMENTS	iii
TABLE OF CONTENTS	iv
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF ABBREVIATIONS	xiii
 1. LITERATURE REVIEW	 1
1.1 Introduction.....	1
1.2 Structural Macromolecules of Cartilage Extracellular Matrix	2
1.2.1 Type II Collagen	2
1.2.2 Type IX Collagen	2
1.2.3 Type XI Collagen	3
1.2.4 Mutations in Collagen Genes.....	4
1.2.5 Cartilage-Specific Proteoglycan (Aggrecan)	4
1.2.6 Other Components of Cartilage Extracellular Matrix	5
1.3 Skeletal Patterning	6
1.3.1 Skeletal Patterning in the Embryonic Limb Bud	6
1.3.2 Skeletal Patterning in the Embryonic Facial Primordia	8
1.4 Regulatory Genes Implicated in Mediating Effects of Early Patterning Signals	8
1.5 Prechondrogenic Mesenchymal Cell Condensation	10
1.5.1 Matrix Molecules Mediating Cell Condensation	10
1.5.2 Role of Gap Junctional Communication in Cell Condensation	13
1.5.3 Role of Cell Shape in Condensation	13
1.5.4 Role of Paracrine/Autocrine Signaling in Condensation.....	14
1.5.5 Role of Transcription Factors in Regulating Cellular Condensation	17
1.6 Negative Modulators of Chondrogenesis	19
1.7 Advantages to an <i>In Vitro</i> Analysis of Chondrogenic Differentiation	21
1.8 Cartilage Maturation and Hypertrophy	23

1.9 Biological Effects of Alcohol	24
1.9.1 Fetal Alcohol-Related Birth Defects	24
1.9.2 Animal Models of Fetal Alcohol Effects	25
1.9.3 Alcohol Metabolism	27
1.9.4 Effects of Alcohol on Embryonic and Cellular Metabolism	28
1.10 Experimental Objectives.....	31
2. MATERIALS AND METHODS	33
2.1 Animals.....	33
2.2 Establishment of Dissociated Cell Cultures from Embryonic Chick Limb and Facial Prechondrogenic Mesenchyme and Differentiated Sternal Chondrocytes	33
2.2.1 Preparation of Cultures from Embryonic Chick Limb Prechondrogenic Mesenchymal Cells.....	33
2.2.2 Establishment of Cultures from Embryonic Chick Facial Mesenchyme Cells	35
2.2.3 Establishment of Cultures from Embryonic Chick Sternal Chondrocytes	35
2.3 Histochemical Detection of Sulfated Glycosaminoglycans (GAGs).....	36
2.3.1 Histochemical Staining with Alcian Blue Dye	36
2.3.2 Quantification of Cartilage GAG Formation by Elution of Alcian Blue Dye with Guanidinium Hydrochloride (GuHCl)	36
2.4 Biochemical Analysis of Sulfated Glycosaminoglycan (GAG) Accumulation.....	37
2.4.1 Labeling of Cultured Cells with $^{35}\text{SO}_4$	37
2.4.2 Measurement of GAG-incorporated $^{35}\text{SO}_4$ in Medium and Cell Layer Fractions of Limb and Facial Mesenchyme Cultures	37
2.4.3 Fluorometric DNA Quantification.....	38
2.5 Analysis of Cellular Proliferation: Labeling of Cultured Cells by ^3H -Thymidine	38
2.6 RNA Analysis	39
2.6.1 RNA Isolation	39
2.6.2 Determination of Relative Amounts of Total Poly (A) ⁺ RNA or 18S rRNA in Individual Samples	40
2.6.3 Analysis of Gene-Specific mRNA Levels by RNA Dot-Blot and Northern Blot Hybridization	41
2.6.4 Generation of cDNA Probes to Cartilage-Characteristic Genes	41
2.7 Polymerase Chain Reaction (PCR)-Based Cloning Strategy	43

2.7.1 cDNA Synthesis.....	43
2.7.2 PCR-Amplification Reactions	43
2.7.3 T/A Cloning	44
2.8 Differential Display Technique	46
2.9 Statistical Analysis.....	47
3. RESULTS	48
3.1 <i>In Vitro</i> Effects of Aliphatic Alcohols on Cartilage Differentiation by Embryonic Limb Mesenchyme.....	48
3.1.1 Ethanol Effects on High-Density Micromass Cultures of Limb Mesenchyme	48
3.1.2 Ethanol Effects on Low-Density Cultures of Limb Mesenchyme	53
3.1.3 Effects of Other Alcohols	56
3.1.4 Effects of Ethanol on Proliferation of High- and Low-Density Cultures of Prechondrogenic Limb Mesenchyme	61
3.1.5 Effect of Medium Serum Concentrations on Ethanol-Responsiveness of Limb Mesenchyme Cell Cultures	64
3.2 Alcohol Effects on <i>In Vitro</i> Cartilage Differentiation by Embryonic Facial Mesenchyme	70
3.2.1 Effects of Ethanol Treatment on Micromass Cultures of Facial Mesenchyme	70
3.2.2 Effects of Tertiary Butanol Treatment on Facial Mesenchyme Cultures	74
3.3 <i>In Vitro</i> Effects of Ethanol Treatment on Differentiated Chondrocytes	78
3.4 Early Changes in Gene Expression Induced by Exposure of Prechondrogenic Limb Mesenchyme to Ethanol.....	79
3.4.1 Effects of Ethanol Treatment on Expression of Putative Chondrogenic Regulatory Genes	79
3.4.2 Effects of Ethanol Treatment on <i>Msx-2</i> Gene Expression.....	86
3.4.3 Effects of Ethanol Treatment on Expression of Hsp Genes	86
3.4.4 "Differential Display": A Technique to Identify Novel Genes that are Preferentially Expressed During Ethanol-Induced Chondrocyte Differentiation	88
3.5 Role of Specific Intracellular Signal Transduction Pathways in Mediating Ethanol's Effects on Chondrogenesis	90
3.5.1 Protein Kinase C (PKC) Inhibitors	92
3.5.2 PKC Downregulators.....	94
3.5.3 Mitogen-Activated Protein (MAP) Kinase Inhibitors	99
3.5.4 Protein Kinase A (PKA) Inhibitors.....	104

3.5.5 Protein Tyrosine Kinase (PTK) Inhibitors.....	107
4. DISCUSSION.....	110
4.1 Ethanol Exposure Elevates <i>In Vitro</i> Chondrogenic Differentiation by Embryonic Limb Mesenchyme.....	110
4.2 Stimulatory Effects of Ethanol are Not Restricted to Prechondrogenic Limb Mesenchyme	112
4.3 Ethanol Directly Influences the Differentiation of Chondrogenic Progenitor Cells	112
4.4 Ethanol's Stimulatory Effects on Chondrogenic Differentiation are a Result of Direct Action of the Alcohol Itself Rather than a Downstream Metabolite	113
4.5 Stimulatory Effects of Ethanol are Not Due to Altered Cellular Proliferation or Viability of Limb Mesenchyme Cells	113
4.6 Ethanol Enhances Chondrogenic Differentiation Only Under Conditions that Permit Some Degree of Spontaneous Chondrogenesis	114
4.7 Ethanol Appears to Act at an Early Point in the Chondrocyte Differentiation Pathway	116
4.8 Ethanol Treatment Does Not Alter <i>Msx-2</i> Gene Expression in Cultures of Prechondrogenic Limb Mesenchyme	119
4.9 Ethanol Treatment Does Not Elicit a Classical Stress Response	120
4.10 The Role of Specific Intracellular Signal Transduction Pathways in Mediating Ethanol's Effects on Chondrogenesis is Unclear	121
4.10.1 Protein Kinase C (PKC) Inhibitors/Downregulators	122
4.10.2 Mitogen-Activated Protein (MAP) Kinase Inhibitors	124
4.10.3 Protein Kinase A (PKA) Inhibitors.....	125
4.10.4 Protein Tyrosine Kinase (PTK) Inhibitors.....	126
4.11 The Potential Relationship of Alcohol's Stimulatory Effects on <i>In Vitro</i> Cartilage Differentiation to its Teratogenic Effects on <i>In Vivo</i> Skeletogenesis.....	126
4.12 Future Directions	128
REFERENCES	134

LIST OF TABLES

Table 1	Effect of medium serum concentration on ethanol-responsiveness of high-density cultures of mesenchyme cells isolated from whole stage 23/24 wing buds.....	69
Table 2	Alcian blue-positive matrix accumulation in cultures of differentiated chondrocytes isolated from caudal and cranial portions of 17 day chick embryo sterna following incubation in control medium or medium supplemented with 1.0% ethanol.	81

LIST OF FIGURES

Figure 1	Effects of various concentrations of ethanol on sulfated glycosaminoglycan (GAG) accumulation and total DNA content in high-density micromass cultures of stage 23/24 chick embryo wing bud mesenchyme.	49
Figure 2	Alcian blue-positive matrix accumulation in ethanol-treated micromass cultures of stage 23/24 whole wing bud mesenchyme.	51
Figure 3	Sulfated GAG accumulation in ethanol-treated micromass cultures prepared from stage 24/25 wing bud distal subridge mesenchyme.	52
Figure 4	Dot-blot analysis examining the effects of ethanol exposure on type II collagen and aggrecan mRNA expression in micromass cultures of whole wing bud mesenchyme.	54
Figure 5	Alcian blue-positive matrix accumulation in ethanol-treated, low-density cultures of whole wing bud mesenchyme.	55
Figure 6	Sulfated GAG accumulation in ethanol-treated, low-density cultures of whole wing bud mesenchyme.	57
Figure 7	Dot-blot analysis examining the effects of ethanol treatment on type II collagen and aggrecan transcript expression in low-density cultures of limb mesenchyme.	58
Figure 8	Alcian blue-positive matrix accumulation in limb mesenchyme cell cultures exposed to various aliphatic alcohols.	59
Figure 9	Influence of various aliphatic alcohols on sulfated GAG accumulation in micromass cultures prepared from whole wing bud mesenchyme.	60
Figure 10	Effects of ethanol and the DNA synthesis inhibitor, cytosine arabinoside, on cellular proliferation in micromass cultures of stage 23/24 whole wing bud mesenchyme.	62
Figure 11	Photomicrographs demonstrating effects of cytosine arabinoside on ability of ethanol to enhance Alcian blue-positive matrix accumulation in micromass cultures of stage 23/24 whole wing bud mesenchyme.	63
Figure 12	Sulfated GAG accumulation in micromass cultures of stage 23/24 whole wing bud mesenchyme cells that have been exposed to ethanol and/or cytosine arabinoside.	65

Figure 13	Effects of ethanol and cytosine arabinoside on ³ H-thymidine uptake in low-density cultures of limb mesenchyme.	66
Figure 14	Photomicrographs demonstrating effects of cytosine arabinoside on ability of ethanol to enhance Alcian blue-positive cartilage matrix accumulation in low-density cultures of whole wing bud mesenchyme.	67
Figure 15	Quantitation of sulfated GAG accumulation in low-density cultures of limb mesenchyme cells that have been exposed to ethanol and/or cytosine arabinoside.....	68
Figure 16	Alcian blue-positive matrix accumulation in ethanol-treated micromass cultures of stage 24 chick embryo facial mesenchyme.	71
Figure 17	Effects of various concentrations of ethanol on sulfated GAG accumulation and total DNA accumulation in micromass cultures of facial mesenchyme.	73
Figure 18	Sulfated GAG accumulation in ethanol-treated micromass cultures of facial mesenchyme.....	75
Figure 19	Dot-blot analysis examining the effects of ethanol exposure on type II collagen and aggrecan mRNA expression in micromass cultures of facial mesenchyme.....	76
Figure 20	Sulfated GAG accumulation in micromass cultures of facial mesenchyme exposed to tertiary butanol.....	77
Figure 21	Photomicrographs demonstrating Alcian blue-positive matrix accumulation in ethanol-treated, monolayer cultures of differentiated sternal chondrocytes.....	80
Figure 22	Northern RNA analysis examining the effects of ethanol treatment on expression of gene transcripts for type II collagen, aggrecan and type X collagen in cultures of differentiated sternal chondrocytes.	82
Figure 23	Dot-blot analysis examining the effects of ethanol exposure on expression of RNA transcripts for <i>Sox-9</i> , <i>BMPR-1B</i> , <i>Ets-2</i> and type II collagen in micromass cultures of limb mesenchyme.	84
Figure 24	Dot-blot analysis examining the effects of ethanol on expression of transcripts for <i>Sox-9</i> and type II collagen in low-density cultures of limb mesenchyme.	85
Figure 25	Effects of ethanol exposure on <i>Erg</i> , TGF- β_3 and <i>Msx-2</i> gene expression in micromass cultures of limb bud mesenchyme.....	87

Figure 26	Dot-blot analysis examining the effects of ethanol treatment on gene transcript expression of Hsp70, Hsp47, and Hsp90 α in micromass cultures of stage 23/24 whole wing bud mesenchyme.	89
Figure 27	Identification of genes differentially expressed at the mRNA level using a novel PCR-based selective cloning strategy, "Differential Display".	91
Figure 28	Photomicrographs demonstrating effects of various Protein Kinase C (PKC) inhibitors on ability of ethanol to enhance Alcian blue-positive cartilage matrix accumulation <i>in vitro</i>	93
Figure 29	Graph illustrating effects of various PKC inhibitors on ability of ethanol to enhance sulfated GAG accumulation in micromass cultures of whole wing bud mesenchyme.	95
Figure 30	Photomicrographs illustrating the influence of various PKC downregulators on ability of ethanol to enhance Alcian blue-positive cartilage matrix accumulation <i>in vitro</i>	96
Figure 31	Graph demonstrating effects of various PKC downregulators on ability of ethanol to enhance sulfated GAG accumulation in micromass cultures of whole wing bud mesenchyme.	97
Figure 32	Effects of PMA on ability of ethanol to enhance type II collagen and <i>Sox-9</i> gene expression in micromass cultures of limb bud mesenchyme.	98
Figure 33	Photomicrographs illustrating the influence of various Mitogen-Activated Protein (MAP) Kinase inhibitors on ability of ethanol to enhance Alcian blue-positive cartilage matrix accumulation <i>in vitro</i>	100
Figure 34	Graph quantifying the influence of various MAP Kinase inhibitors on ability of ethanol to enhance sulfated GAG accumulation in micromass cultures of limb mesenchyme.	101
Figure 35	Photomicrographs demonstrating the effect of Wortmannin on ability of ethanol to enhance Alcian blue-positive matrix accumulation <i>in vitro</i>	102
Figure 36	Graph illustrating the effect of Wortmannin on ability of ethanol to enhance sulfated GAG accumulation in micromass cultures of limb mesenchyme.	103
Figure 37	Photomicrographs illustrating effects of various Protein Kinase A (PKA) inhibitors on ability of ethanol to enhance Alcian blue-positive cartilage matrix accumulation <i>in vitro</i>	105
Figure 38	Graph quantifying effects of various PKA inhibitors on ability of ethanol to enhance sulfated GAG accumulation in micromass cultures of limb bud mesenchyme.	106

Figure 39	Photomicrographs demonstrating the influence of various Protein Tyrosine Kinase (PTK) inhibitors on ability of ethanol to enhance Alcian blue-positive cartilage matrix accumulation <i>in vitro</i>.	108
Figure 40	Graph illustrating the influence of various PTK inhibitors on ability of ethanol to enhance sulfated GAG accumulation in micromass cultures of limb bud mesenchyme.	109

LIST OF ABBREVIATIONS

A.B.	alcian blue
ADH	alcohol dehydrogenase
AER	apical ectodermal ridge
AIDH	aldehyde dehydrogenase
ANOVA	analysis of variance
A-P	anterior-posterior
BME	β -mercaptoethanol
BMP	bone morphogenetic protein
BMPR	BMP receptor
cAMP	cyclic adenosine monophosphate
<i>cho</i>	autosomal recessive <i>chondrodysplasia</i> mutation in mice
CMP	cartilage matrix protein
CNS	central nervous system
COL2A1	gene encoding the alpha-1 polypeptide chains of type II collagen
COL11A1	gene encoding the alpha-1 polypeptide chain of type XI collagen
COMP	cartilage oligomeric matrix protein
CPC	cetylpyridinium chloride
CREB	cAMP response element binding protein
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
D-V	dorsal-ventral
<i>E. coli</i>	<i>Escherichia coli</i> bacteria
EDTA	ethylenediamine tetraacetic acid-disodium salt
EGF	epidermal growth factor
FACIT	fibril associated collagens with interrupted triple helices
FAE	fetal alcohol-related effects
FAS	fetal alcohol syndrome
FBS	fetal bovine serum
FGF	fibroblast growth factor
FN	fibronectin

GAG	glycosaminoglycan
GDF	growth and differentiation factor
GuHCl	guanidinium hydrochloride
HA	hyaluronan
HBSS	Hank's balanced salt solution
Hsp	heat shock protein
IC₅₀	concentration to produce 50% inhibition
IGF	insulin-like growth factor
kDa	kilodalton
K_i	inhibition constant
LB	Luria Bertani medium
MAP	mitogen-activated protein
MES	(2[Nmorpholino]ethanesulfonic acid
MOPS	(3[Nmorpholino]propanesulfonic acid
NCAM	neural cell adhesion molecule
OIL	octylindolactam V
PCR	polymerase chain reaction
P-D	proximal-distal
PG	prostaglandin
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
RA	retinoic acid
RAR	RA receptor
RT-PCR	reverse transcriptase PCR
RXR	retinoid X receptor
SDS	sodium dodecyl sulfate
<i>se</i>	<i>short-ear</i> mutant in mice
Shh	sonic hedgehog
SSC	sodium chloride/sodium citrate solution
TE	Tris/EDTA
TFB	transformation buffer
TGF-β	transforming growth factor-beta
ZPA	zone of polarizing activity

1. LITERATURE REVIEW

1.1 Introduction

Hyaline cartilage plays an essential role during skeletal development (reviewed in Cancedda et al., 1995; Poole, 1997). It forms the initial cartilaginous models of bones of the appendicular skeleton, sternum, vertebral column, pelvis, and components of the craniofacial skeleton and base of the skull. These anlagen are later replaced by bone during endochondral ossification. Cartilage remains, however, as articular cartilage on diarthrodal joint surfaces of bones where it functions as a low-friction, compressive, load-bearing tissue, and as growth plate cartilage between the epiphyses and diaphyses of long bones where it acts as an intermediate tissue in bone growth and repair.

The unique biomechanical properties of cartilage stem mainly from the nature and predominance of extracellular matrix macromolecules (chiefly, glycosaminoglycans and collagen) that have been secreted by chondrocytes. Polysaccharide glycosaminoglycans (GAGs), which are usually found covalently linked to protein in the form of proteoglycans, and hyaluronan (HA) make up the highly hydrated, gel-like ground substance. Collagen fibrils, containing type II collagen as the major component and smaller amounts of types IX and XI collagens, provide tensile strength and help to organize the extracellular matrix.

Three distinct embryonic progenitor cell lineages contribute to the cartilaginous skeleton. The neural crest gives rise to the chondrocytes of the facial skeleton and anterior cranial base, the sclerotome generates the axial skeleton and ribs, and the lateral plate mesoderm forms the appendicular skeleton and sternum. Regardless of their specific embryonic origin, however, the differentiation of mesenchymal cartilage progenitor cells is characterized by similar changes in cell morphology, cell proliferation, and extracellular matrix production. Foremost in the steps leading to the acquisition of the characteristic chondrocyte phenotype is an initial aggregation or condensation of prechondrogenic mesenchyme cells. The resulting cell-cell interactions trigger the onset of overt chondrocyte differentiation which is characterized by a switch from a fibroblastic morphology to a spherical cell shape, and by the secretion of an extracellular matrix composed predominantly of type II collagen and aggrecan.

1.2 Structural Macromolecules of Cartilage Extracellular Matrix

1.2.1 Type II Collagen

Type II collagen is the major protein of hyaline cartilage collagen fibrils (Mendler et al., 1989) and is comprised of three identical polypeptide subunits, called $\alpha 1$ (II) chains, that are the encoded products of a single gene, COL2A1 (Bornstein and Sage, 1980; Eyre, 1980; reviewed in Upholt, 1989). COL2A1 transcripts are alternatively spliced to produce mRNAs that either include (type IIA mRNA) or exclude (type IIB mRNA) an exon encoding a 70 amino acid, cysteine-rich globular domain in the amino-propeptide (Ryan and Sandell, 1990; Metsaranta et al., 1991; Nah and Upholt, 1991; Lui et al., 1995). Low levels of type IIA collagen mRNA are expressed in both prechondrogenic tissues (Kosher et al., 1986a), and in nonchondrogenic tissues including the vitreous of the eye, the nucleus pulposus of intervertebral disks, and embryonic chick primary corneal stroma (von der Mark et al., 1977; Bornstein and Sage, 1980; Eyre, 1980). In addition, it is present within the basement membranes of several embryonic epithelia (Thorogood et al., 1986; Thorogood, 1988; Fitch et al., 1989; Kosher and Solursh, 1989). As prechondrogenic mesenchyme cells aggregate and initiate chondrogenesis, however, there is a switch from type IIA collagen mRNA expression to type IIB collagen mRNA expression (Ng et al., 1993; Sandell et al., 1994). Thereafter, there is a progressive increase in cytoplasmic levels of type IIB collagen mRNA which parallels the progressive accumulation of extracellular cartilage matrix.

1.2.2 Type IX Collagen

Type IX collagen is comprised of three genetically distinct polypeptide subunits called $\alpha 1$ (IX), $\alpha 2$ (IX) and $\alpha 3$ (IX) chains. Each of these subunits contain three helical, collagenous domains that are separated and flanked by nonhelical domains (van der Rest et al., 1985). It is not capable of forming fibrils by itself, and is therefore referred to as a nonfibrillar collagen or as a fibril associated collagen with interrupted triple helices (FACIT). Instead, type IX collagen is located in a periodic manner along the surface of collagen fibrils where it projects its amino-terminal, triple-helical domain as an arm into the perifibrillar matrix (Irwin and Mayne, 1986; Vaughan et al., 1988). In cartilage, this arm continues into a globular domain that is formed by the amino-terminal portion of the $\alpha 1$ (IX) polypeptide chain and has been shown to interact with cartilage proteoglycans (Vasios et al., 1988). Thus, it has been suggested that type IX collagen molecules on the fibrillar surface may provide a molecular link between the

fibrils and the surrounding matrix. A second polypeptide chain, $\alpha 2$ (IX), carries a single covalently-bound GAG chain, and as such, type IX collagen is also classified as a proteoglycan (Bruckner et al., 1985; Vaughan et al., 1985; Huber et al., 1986; Konomi et al., 1986; McCormick et al., 1987).

Type IX collagen is a major component of cartilage collagen fibrils, together with the fibrillar collagens type II and XI (Mendler et al., 1989). However, the expression of type IX is not limited to cartilage. Rather, like type II collagen, type IX collagen is also expressed in the vitreous of the eye and the nucleus pulposus of the intervertebral disks (Ayad et al., 1982; Wright and Mayne, 1988), and is also present under several embryonic epithelia (Fitch et al., 1988; Svoboda et al., 1988).

Interestingly, the $\alpha 1$ (IX) gene has 2 alternative promoters and transcription start sites (Nishimura et al., 1989). The two promoters are located 20 kb apart, flanking a series of exons that encode the globular domain at the amino end of type IX collagen molecules. In cartilage, the most 5' promoter is used and the amino-terminal globular domain is expressed. In contrast, in embryonic corneal epithelium the most 3' transcription start site is used, such that the resulting mRNA is approximately 700 nucleotides shorter and the globular domain is not expressed. Modulation of the expression of the amino-terminal globular domain of type IX collagen may represent a mechanism by which cells in different tissues can modulate the interaction of collagen fibrils with other matrix components such as proteoglycans.

1.2.3 Type XI Collagen

Type XI collagen is expressed in embryonic hyaline cartilages where it constitutes approximately 10% of the total collagen (Eyre and Wu, 1987). It is a heterotrimeric molecule that is composed of three polypeptide subunits called $\alpha 1$ (XI), $\alpha 1$ (XI) and $\alpha 3$ (XI) chains (Morris and Bachinger, 1987). The $\alpha 1$ (XI) and $\alpha 2$ (XI) chains are encoded by distinct genes. However, the $\alpha 3$ (XI) chain is believed to be derived from the COL2A1 gene like the $\alpha 1$ (II) polypeptide chains of type II collagen (Burgeson et al., 1982). Although it is known to be a component of cartilage collagen fibrils, together with type II and IX collagens, the precise location of type XI collagen within the fibril has not yet been definitively determined. Evidence suggests, however, that it may form a central core, and play a role in the regulation of fibrillar diameter (Eyre and Wu, 1987; Linsenmayer et al., 1993).

1.2.4 Mutations in Collagen Genes

Given the importance of collagen fibrils as elements of high tensile strength in cartilage extracellular matrix, it is not surprising that mutations in the genes encoding the polypeptide chains of collagen types II, IX and XI cause numerous forms of osteochondrodysplasias characterized by a loss of the normal mechanical properties of the cartilage tissue (Lee et al., 1989; Vissing et al., 1989; Ahmad et al., 1991; Bogaert et al., 1992; Winterpacht et al., 1993; Vikkula et al., 1995; Muragaki et al., 1996). Some of the mutations also cause alterations in chondrocyte differentiation and maturation, suggesting that normal chondrocyte differentiation may depend on the presence of a normal extracellular matrix. For example, *Coll11A1* has been identified as the candidate gene in autosomal recessive chondrodysplasia (*cho*) in mice, a defect characterized by severe disproportionate dwarfism in homozygotes (Li et al., 1995). The *Coll11A1* mutation not only results in a premature termination of the translation of the $\alpha 1$ (XI) polypeptide, but also causes a dramatic reduction in the level of $\alpha 1$ (XI) mRNA (Li et al., 1995). This suggests that the *cho* phenotype may be caused by a loss of $\alpha 1$ (XI) collagen, rather than synthesis of significant amounts of a truncated $\alpha 1$ (XI) collagen polypeptide. In the absence of normal $\alpha 1$ (XI) chains, no significant amounts of type XI collagen molecules are assembled in *cho* cartilage, which may explain the loss of the normal mechanical properties of the tissue (Li et al., 1995). Interestingly, chondrocyte maturation and hypertrophy is also severely compromised in *cho* mice, although the mechanisms through which this occurs are not yet understood (Olsen, 1996). Analyses of human and mouse collagen type II mutations suggest, however, that not all mutations that cause severe abnormalities in matrix cohesivity have the effect on chondrocyte maturation seen in *cho* (Garofalo et al., 1991; Metsaranta et al., 1992; Li et al., 1995; Olsen, 1995). As such, it is possible that collagen type XI may play a more specific role in chondrocyte differentiation.

1.2.5 Cartilage-Specific Proteoglycan (Aggrecan)

Aside from collagens, the other predominant component of cartilage extracellular matrix is a large keratan sulfate/chondroitin sulfate-rich proteoglycan, termed aggrecan (Hassell et al., 1986; Sandell, 1987; Ruoslahti, 1988). The sulfated GAG chains of aggrecan are highly negatively charged, such that osmotically active cations (e.g., Na^+) are attracted and large amounts of water are subsequently drawn into the matrix. This creates a swelling pressure, or turgor, that enables the cartilage extracellular matrix to withstand compressive forces. In addition to providing mechanical support to the avascular cartilage tissue, the sulfated GAG chains also form

a porous, hydrated gel that allows the rapid diffusion of oxygen, nutrients, metabolites, and hormones to the embedded chondrocytes.

Aggrecan monomers assemble in the extracellular space into large aggregates that are noncovalently bound through their core proteins to molecules of hyaluronan (HA), a long unbranched, non-sulfated GAG. As many as 100 proteoglycan monomers may bind to a single HA chain, producing a giant multimeric complex. Link protein is believed to stabilize the binding between HA and the aggrecan monomers in cartilage matrix since it contains domains for interaction with both HA (Doege et al., 1986; Goetinck et al., 1987; Kiss et al., 1987; Rhodes et al., 1988) and the amino terminus of the aggrecan core protein (Doege et al., 1990).

The expression of aggrecan gene transcripts appears to be largely limited to cartilaginous tissues (Sai et al., 1986). Unlike type II collagen mRNA, no aggrecan RNA is detectable in prechondrogenic mesenchyme. Rather, aggrecan mRNAs begin to accumulate at the onset of chondrogenic differentiation, coincident with the crucial prechondrogenic mesenchymal cell condensation phase and prior to the deposition of a cartilaginous extracellular matrix. Following condensation, there is a progressive rise in both aggrecan and type II collagen RNA levels which parallels the progressive accumulation of cartilage matrix (Kosher et al., 1986b).

1.2.6 Other Components of Cartilage Extracellular Matrix

Several other macromolecules have also been identified as minor components of cartilage matrix, although their precise functions have not yet been determined. Only a few will be briefly mentioned here, such as cartilage matrix protein (CMP), biglycan, decorin, and cartilage oligomeric matrix protein (COMP). CMP is expressed only in mature, well-differentiated cartilage just prior to the appearance of type X collagen in hypertrophic chondrocytes (Paulsson and Heinegard, 1984; Argraves et al., 1987; Stirpe and Goetinck, 1989). It has been suggested that CMP may serve as a bridge between the collagen fibril and the ternary complex of aggrecan monomer, link protein and HA (Paulsson and Heinegard, 1984; Argraves et al., 1987; Goetinck et al., 1990). Biglycan and decorin, on the other hand, have been detected in both developing and mature cartilages (Rosenberg et al., 1985; Bianco et al., 1990). Both of these proteoglycans have been shown to bind to collagens (Scott, 1988; Schonherr et al., 1995), leading to the suggestion that biglycan and decorin may act to regulate the formation of collagen fibrils (Vogel and Trotter, 1987). COMP is another molecule that has been identified in hyaline cartilage (Franzen et al., 1987; Hedbom et al., 1992). Although its function is

unknown, COMP has been shown to act as a cell binding protein, and as such, may play a role in cell-matrix interactions (DiCesare et al., 1994).

1.3 Skeletal Patterning

A critical role of cartilage differentiation in skeletogenesis is suggested by the fact that the phases of prechondrogenic mesenchymal cell proliferation, condensation, and initial cartilage matrix deposition correspond to the period of maximum embryonic vulnerability to teratogenic agents (e.g., alcohol, retinoic acid) that induce skeletal deformities *in vivo*. Accordingly, elucidation of the molecular events regulating embryonic cartilage differentiation is fundamental to understanding both normal skeletogenesis and the etiology of congenital skeletal anomalies. My discussion will focus on molecules that are involved in specifying the identity and shape of components of the skeleton, and in regulating mesenchymal cell "condensation" that occurs prior to overt cartilage differentiation. The embryonic limb bud has been used extensively to study skeletal pattern formation and cartilage differentiation. As such, I will focus my discussion on epithelial-mesenchymal, cell-cell and cell-matrix interactions occurring in the developing limb. Brief mention will be given to interactions regulating cartilage formation in the facial primordia as well.

1.3.1 Skeletal Patterning in the Embryonic Limb Bud

The muscular, skeletal, and connective tissues of the vertebrate tetrapod limb develop from undifferentiated mesenchymal cells which arise from two distinct progenitor cell lineages (Sasse et al., 1984). All skeletal muscle fibers of the limb differentiate from premyogenic mesenchyme that migrates into the embryonic limb primordium from adjacent somites (Chevallier et al., 1977; Christ et al., 1977). In contrast, the cartilage, bone, and other connective tissues of the limb differentiate from somatopleural (lateral plate) mesoderm of the prospective limb-forming regions of the flank (Wachtler et al., 1981). Distal outgrowth of the embryonic limb is directed by the epithelium of the apical ectodermal ridge (AER) located at the distal tip of the limb bud, which maintains the underlying chondrogenic and myogenic progenitor cells in a proliferative, undifferentiated state (Saunders, 1948; Summerbell, 1974). Overt histogenesis of cartilage and muscle tissues is initiated as the proliferating limb mesenchyme cells emerge from the "progress zone" or "subridge region" of AER influence (Stark and Searls, 1973). Thus, histogenesis of skeletal elements proceeds in a proximal to distal sequence, beginning with the humerus or femur, and ending with the formation of the distal phalanges. In addition to its role in proximal-distal (P-D)

patterning, ectodermal signaling has also been implicated in patterning of the dorsal-ventral (D-V) limb axis. Since reversal of the ectoderm 180° about its D-V axis results in an inversion of the D-V polarity of limb skeletal structures, it would appear that ectodermal signals specify fate along this axis (MacCabe et al., 1974; Pautou, 1977). Establishment of the anterior-posterior (A-P) polarity of the limb involves mesodermal cells of the zone of polarizing activity (ZPA), a region at the posterior margin of the limb bud. Transplantation of ZPA tissue to the anterior border of a limb bud results in a mirror-image duplication of the limb (Saunders and Gasseling, 1968).

A number of signaling molecules have now been identified that act cooperatively in determining limb pattern (Laufer et al., 1994; Niswander et al., 1994; Parr and McMahon, 1995; Yang and Niswander, 1995). For example, the outgrowth-promoting activity of the AER can be functionally replaced by the local application of peptides of the fibroblast growth factor (FGF) family (Niswander et al., 1993; Fallon et al., 1994), several of which are expressed endogenously in the AER (Suzuki et al., 1992; Niswander and Martin, 1992; Niswander and Martin, 1993a). Therefore, it seems likely that one or more of these factors is responsible for regulating the proximal-distal (P-D) outgrowth of the developing limb, and for maintaining the chondrogenic and myogenic cells of the progress zone in an undifferentiated state. Another putative ectodermal signaling molecule is Wnt-7A, a secreted factor that is expressed in the dorsal ectoderm and is believed to specify cell fate along the D-V axis (Dealy et al., 1993; Parr et al., 1993). Evidence to support this conclusion has come from the demonstration that ectopic Wnt-7A can dorsalize ventral mesoderm in the distal portion of the limb bud (Riddle et al., 1995; Vogel et al., 1995). In contrast, loss of Wnt-7A function following targeted gene disruption leads to the acquisition of ventral characteristics in dorsal limb regions (Parr and McMahon, 1995). A-P polarity within the developing limb is believed to be specified by the secreted factor, Sonic hedgehog (Shh). Evidence to support this conclusion has come from the demonstration that Shh expression colocalizes with ZPA activity in the developing limb bud (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993). Moreover, Shh-expressing cells implanted along the anterior margin of the limb bud can mimic transplanted ZPA tissue, and induce a mirror-image duplication of the limb (Riddle et al., 1993; Chang et al., 1994; Lopez-Martinez et al., 1995). The vitamin A metabolite, retinoic acid (RA), is also believed to play a crucial role in regulating skeletal patterning along the A-P axis of the limb. Retinoic acid-soaked beads applied locally to the anterior margin of the developing limb activates Shh expression and induces mirror-image limb duplications by inducing the formation of an ectopic ZPA (Noji et al., 1991; Wanek et al., 1991;

Riddle et al., 1993). In contrast, local application of inhibitors of retinoid synthesis or activity to the flank of the chick embryo prevents the induction of Shh expression (Helms et al., 1996; Stratford et al., 1996).

1.3.2 Skeletal Patterning in the Embryonic Facial Primordia

Unlike the limb skeleton, the vertebrate facial skeleton (frontal bone, nasal septum, maxilla, palate, mandible, and hyoid apparatus) differentiates from neural crest-derived ectomesenchyme cells that invade the embryonic frontonasal, maxillary, mandibular, and hyoid prominences (LeLievre, 1978; Noden, 1983; Couly et al., 1993). As in the limb bud, skeletal patterning in the facial primordia also appears to involve an extensive set of epithelial-mesenchymal interactions (Wedden, 1987). Indeed, it has been shown that if the mandibular epithelium is removed, the underlying mesenchyme halts its P-D outgrowth and precociously differentiates into cartilage and bone (Wedden, 1987; Richman and Tickle, 1989; Hall and Coffin-Collins, 1990; Mina et al., 1994). Thus, it appears that the role of mandibular epithelium in the outgrowth of the developing mandible is similar to the effects of the AER in the developing limb. However, unlike the developing limb, where the mechanisms of morphogenesis and pattern formation are relatively well characterized, less information is available regarding these processes in the developing facial primordia. More recently, however, it has been demonstrated that embryonic facial primordia contain signaling centers that are analogous to the ZPA of the limb that participate in craniofacial growth and patterning. Indeed, it has been demonstrated that the epithelial layer of craniofacial primordia expresses Shh, and exhibits polarizing activity when grafted into a chick wing bud (Helms et al., 1997).

1.4 Regulatory Genes Implicated in Mediating Effects of Early Patterning Signals

Although early coordinated signals provide positional information to cells within an embryonic region, these signals must be interpreted in order to form the appropriate skeletal structures. Mounting evidence suggests that key regulatory genes play a crucial role in mediating the instructions encoded by early patterning signals. Examples of these key regulatory genes are the highly-conserved homeobox-containing genes that encode putative or known transcription factors. Indeed, the expression of one such homeobox-containing gene, *Lmx-1*, is induced in dorsal mesoderm by the dorsalizing ectoderm-derived signal, Wnt-7A (Riddle et al., 1995; Vogel et al., 1995).

Another important group of genes in this regard are the clustered *Hox* genes (reviewed in Krumlauf, 1994). The expression domains of 5' members of the *Hox D* gene cluster (d9 - d13), for example, are initially established in a nested set centered around the posterior of the developing limb (Dolle et al., 1989). These genes can be ectopically activated by the combined influence of Shh and FGF, suggesting that these signaling molecules play an endogenous role in the regulation of *Hox D* genes (Nelson et al., 1996). *Evx-1* is a member of the *Hox A* gene family that has been shown to be expressed in progress zone mesoderm immediately after the formation of the AER. Its expression can also be induced following exposure to exogenous FGFs (Niswander and Martin, 1993b).

The non-clustered homeobox-containing *Msx* genes are also thought to play an important role in pattern formation during vertebrate development. *Msx-1* is expressed in the highly proliferative and undifferentiated cells of the progress zone mesoderm of the developing chick limb bud, suggesting its possible involvement in mediating the P-D outgrowth of the mesoderm in response to the AER (Nohno et al., 1992; Ros et al., 1992; Coelho et al., 1993a,b). *Msx-2*, on the other hand, is expressed in the AER, which suggests that it may play a role in regulating some aspect of AER activity (Coelho et al., 1991; Nohno et al., 1992). Both *Msx-1* and *Msx-2* are expressed in the anterior nonchondrogenic region of the developing limb, suggesting their potential involvement in specifying anterior positional identity (Coelho et al., 1991; Yokouchi et al., 1991). *Msx-1* and *Msx-2* are also co-expressed in the posterior necrotic zone and in the interdigital mesoderm at later stages of development (Coelho et al., 1991; Yokouchi et al., 1991; Coelho et al., 1993a). Thus, both of these genes may also be involved in developmentally programmed cell death. Together, these studies suggest that *Msx-1* and *Msx-2* may act cooperatively to inhibit cell proliferation and promote apoptosis in regions in which they are expressed, thereby restricting the outgrowth and formation of skeletal elements to the progress zone mesoderm (Ferrari et al., 1998).

Members of another highly-conserved family of genes, the paired-box (*Pax*) gene family, encode transcription factors that have also been implicated as regulators of skeletal pattern formation (Gruss and Walther, 1992). *Pax1* and *Pax3*, for example, are expressed in spatiotemporal patterns consistent with their involvement in the development of the vertebral column and in neural crest cell patterning, respectively (Deutsch et al., 1988). Moreover, mutations in the mouse *Pax1* gene in mice cause the "undulated" (*un*) phenotype characterized by defects of the vertebral column and a kinky tail (Balling et al., 1988). Mutations in the *PAX3* gene in humans have been

implicated in Waardenburg syndrome, a dominantly inherited condition that results in a spectrum of craniofacial malformations (Tassabehi et al., 1992).

1.5 Prechondrogenic Mesenchymal Cell Condensation

The current data on the regulation of skeletogenesis suggest a two-step model for the specification of the pattern and shape of the embryonic skeleton (Erlebacher et al., 1995). First, the patterned expression of homeobox genes, etc. confers information to cells of early mesenchymal lineages that specifies the overall pattern of the skeleton and the archetypal shape and identity of the individual skeletal elements. Second, the shape of a skeletal element is further defined through the local regulation of mesenchymal cell condensation by secreted autocrine/paracrine factors.

1.5.1 Matrix Molecules Mediating Cell Condensation

Cell condensation is a critical stage in chondrogenic differentiation during which prechondrogenic mesenchyme cells in the central core of skeletal primordia become closely juxtaposed to one another. This process is mediated through the adhesive interactions of cell surface and pericellular matrix components. Several extracellular matrix molecules have been implicated in these interactions, including the glycosaminoglycan, hyaluronan (HA); large chondroitin-sulfate proteoglycans, PG-M and versican; fibronectin, tenascin, and syndecan.

Prior to the condensation process in the developing limb, prechondrogenic mesenchyme cells are separated by an extensive extracellular matrix, with HA being the predominant glycosaminoglycan. However, with the initiation of cellular condensation, HA distribution becomes patterned. Although it remains uniformly distributed in the limb periphery, there is a progressive decline in HA within prechondrogenic cell aggregates (Kosher et al., 1981; Singley and Solursh, 1981; Knudson and Toole, 1985). Indeed, the large amount of extracellular HA accumulated by early prechondrogenic mesenchyme cells is believed to prevent the cell-cell and/or cell-matrix interactions that are necessary to trigger chondrogenesis (Toole, 1972; Kosher et al., 1981; Knudson and Toole, 1985). Despite this decline, however, HA is still required for early adhesive interactions. Evidence to support this conclusion has come from the demonstration that the onset of condensation coincides with the appearance of HA-binding sites on limb mesenchyme cells (Knudson and Toole, 1987). Moreover, perturbations of HA-cell interactions result in a delay of aggregate formation and chondrogenic differentiation *in vitro* (Maleski and Knudson, 1996). Therefore, the initiation of cellular condensation is believed to involve both the formation of cell aggregates by HA cross-bridging of

adjacent cells, and the removal of excess HA within prechondrogenic cell aggregates. HA's role in the formation of large aggregates of aggrecan monomers that give cartilage extracellular matrix its unique mechanical properties has already been discussed.

Fibronectin (FN), a large extracellular glycoprotein, is also evenly distributed throughout the intercellular space of mesenchyme prior to condensation (Dessau et al., 1980; Melnick et al., 1981; Kosher et al., 1982; Tomasek et al., 1982). It then accumulates in the region of incipient chondrogenesis, and reaches its maximum density just prior to overt cartilage differentiation (Thorogood and Hinchliffe, 1975; Kulyk et al., 1989a). As such, it has been suggested that FN may regulate the onset of chondrogenesis by actively promoting prechondrogenic aggregate formation. More recently, it has been observed that FN gene transcripts are alternatively spliced during the course of chondrogenic differentiation (Bennett et al., 1991; Gehris et al., 1996). All FN gene transcripts from prechondrogenic mesenchyme contain exons III A, B and V, whereas those from mature, differentiated cartilage contain only B and V, but not A. These changes in FN splicing occur at the mRNA level both *in vivo* and *in vitro*, just after the condensation event (Gehris et al., 1996), and correspond to changes in isoform expression at the protein level (Gehris et al., 1997). Interestingly, the addition of antibodies against the region encoded by exon IIIA prior to condensation disrupts cell aggregation, suggesting that FN-induced condensation may be mediated, at least in part, by repeat IIIA (Gehris et al., 1997; Mackie and Murphy, 1998). This may explain why the plasma FN used in earlier studies, which lacks type III repeats A and B, inhibited chondrogenesis (Swalla and Solursh, 1984).

Tenascin C has also been shown to be associated with chondrogenic tissues undergoing differentiation. It is present in prechondrogenic mesenchyme cell condensations, but not in the surrounding nonchondrogenic mesenchyme. It progressively disappears with the accumulation of mature cartilage matrix (Mackie et al., 1987). Tenascin C is retained, however, in the perichondrium, which provides a source of cells to differentiate into chondroblasts (Mackie et al., 1987). These studies suggest that, like FN, tenascin C may promote mesenchyme cell condensation and subsequent chondrogenic differentiation. Indeed, the observation that limb mesenchyme cell cultures plated on a tenascin C substrate exhibited greater cartilage matrix accumulation than control cultures supports this hypothesis (Mackie et al., 1987). Moreover, tenascin C inhibited the attachment of cultured mesenchyme cells to fibronectin-coated tissue culture plates, thereby allowing the cells to round up, and initiate chondrogenesis (Mackie et al., 1987). More recently, it has been found that tenascin C is also subjected to alternative slicing events during chondrogenic

differentiation (Mackie and Murphy, 1998). Indeed, tenascin 190 kilodalton (kDa), 200 kDa and 230 kDa isoforms (tenascin 230 containing the greatest number of FNIII repeats and tenascin 190 containing the least) are each expressed by prechondrogenic mesenchyme cells. However, as chondrogenic differentiation proceeds, tenascin 230 kDa expression progressively declines (Mackie and Murphy, 1998).

It has also been demonstrated that expression of the heparan-sulfate proteoglycan, syndecan-3, is progressively elevated during the condensation phase of chondrogenesis both *in vivo* and *in vitro* (Gould et al., 1992; Gould et al., 1995). As such, it has been suggested that syndecan-3 may play a role in regulating the onset of chondrogenesis. Functional evidence to support this conclusion has come from the demonstration that antibodies directed against syndecan-3 inhibit cartilage differentiation *in vitro* (Seghatooleslami and Kosher, 1996). As chondrogenic differentiation proceeds, syndecan-3 expression declines (Gould et al., 1992). In contrast, the expression of another member of this proteoglycan family, syndecan, declines in limb mesenchyme cell aggregates prior to overt chondrocyte differentiation *in vitro* and *in vivo* (Solursh et al., 1990).

PG-M, a large chondroitin-sulfate proteoglycan, and versican, an alternatively-spliced form of PG-M (Shinomura et al., 1993), have been shown to be expressed at high levels in condensing mesenchyme. Both PG-M and versican gene expression are subsequently downregulated during chondrogenic differentiation (Shinomura et al., 1993; Zhang et al., 1998), coincident with the activation of aggrecan synthesis. Thus, it has been suggested that PG-M and versican may play an active role in the mesenchymal aggregation process. Alternatively, these molecules may simply represent specific secretory products of the prechondrogenic mesenchyme which must be removed at the cellular aggregation phase. Indeed, type I collagen is also expressed at high levels in prechondrogenic mesenchyme, and is subsequently downregulated prior to chondrogenic differentiation (Kosher et al., 1986a).

Several cell surface adhesion molecules have also been implicated as regulators of prechondrogenic cell condensation. For example, both NCAM and N-cadherin have been shown to be expressed in prechondrogenic condensations (Oberlender and Tuan, 1994; Tavella et al., 1994) and are subsequently downregulated upon chondrogenic differentiation. Moreover, exposure of cultured prechondrogenic mesenchyme to antibodies against these cell adhesion molecules inhibits cell aggregation whereas overexpression of these molecules enhances cell condensation and chondrogenic differentiation (Widelitz et al., 1993; Oberlender and Tuan, 1994).

1.5.2 Role of Gap Junctional Communication in Cell Condensation

Cellular condensation is believed to enable crucial cell-cell interactions among prechondrogenic mesenchyme cells that trigger the onset of overt chondrogenic differentiation and activate cartilage-specific gene expression (Kosher, 1983). Indeed, Rodgers et al. (1989) have demonstrated that the intercellular transfer of cAMP, a molecule implicated in the regulation of cartilage differentiation, occurs only under high-density culture conditions which allow close cellular juxtapositions and interactions (Frenz et al., 1989; Kulyk et al., 1989b; Jiang et al., 1993). The exact nature of these cellular interactions remains unclear. Evidence suggests, however, that gap junctions may play an important role in this process since extensive gap junctional communication has been found to occur between condensing prechondrogenic limb mesenchyme cells in culture, but not between the nonchondrogenic cells destined to form the connective tissues of the limb (Coelho and Kosher, 1991).

1.5.3 Role of Cell Shape in Condensation

A change in the shape of mesenchyme cells from a flattened, fibroblastic morphology to a rounded configuration is also believed to be an important factor in the cellular condensation process (Archer et al., 1982; Zanetti and Solursh, 1984; Daniels and Solursh, 1991). Indeed, single mesenchyme cells, which normally do not differentiate into chondrocytes spontaneously, will undergo chondrogenesis and express cartilage-specific genes under conditions in which the cells are maintained in a rounded configuration, such as in suspension culture, or in three-dimensional matrices of agarose, soft agar or collagen gels (Solursh and Reiter, 1975; Solursh et al., 1982; Solursh et al., 1986). Culture under these conditions not only promotes the initiation of chondrogenesis by prechondrogenic mesenchyme cells, but also allows fibroblast-like dedifferentiated chondrocytes to revert to a spheroidal chondrocyte phenotype (Castagnola et al., 1986). Further evidence of the importance of a rounded cell shape in chondrogenic differentiation has come from the demonstration that when single limb mesenchyme cells *in vitro* are treated with cytochalasin D, which disrupts actin microfilaments, the cells transiently round up, then initiate chondrogenesis (Zanetti and Solursh, 1984). In contrast, culture conditions (e.g., fibronectin-coated tissue culture dishes) that promote cell flattening and the stable attachment of mesenchyme cells to the extracellular matrix inhibit the initiation of chondrogenesis (Swalla and Solursh, 1984; Zanetti and Solursh, 1984).

1.5.4 Role of Paracrine/Autocrine Signaling in Condensation

Although considerable insight has been gained into the extracellular molecules that mediate prechondrogenic condensation, as well as the conditions that enable crucial cell-cell interactions among prechondrogenic mesenchyme cells, much less is known about the paracrine/autocrine signals that regulate this process. Prostaglandins (PGs), cAMP, and growth factors of the Transforming Growth Factor-Beta (TGF- β) and Bone Morphogenetic Protein (BMP) families, are amongst the molecules that have been implicated in the cell signaling events that initiate cartilage differentiation.

Prostaglandins, a family of eicosanoid fatty acids derived from arachidonate metabolism, have been implicated as being important factors in the differentiation of chondroblasts into chondrocytes. The effects of prostaglandins on chondrogenesis appear to be mediated by activation of the intracellular cAMP second messenger system (Gay and Kosher, 1984; Biddulph et al., 1988). Evidence to support this hypothesis comes from various *in vitro* experiments in which exposure to either exogenous PGE₂ (Kosher and Walker, 1983; Chepenik et al., 1984; Biddulph et al., 1988), or exogenous cAMP analogues (Ahrens et al., 1977; Solursh et al., 1981a) dramatically enhances the level of cartilage matrix accumulation by prechondrogenic limb mesenchyme cells. In addition, an increase in the synthesis of both endogenous PGE₂ (Gay and Kosher, 1985) and endogenous cAMP (Solursh et al., 1979, Ho et al., 1982; Biddulph et al., 1984) has been shown to correlate temporally with the initial phase of prechondrogenic cell condensation that precedes overt cartilage differentiation. cAMP responds maximally to PGE₂ at this time, enhancing the expression of cartilage-specific genes, type II collagen and aggrecan (Biddulph et al., 1984). More recently, it has been shown that cAMP (Gonzalez and Montminy, 1989; Lee and Chuong, 1997) stimulates chondrogenic differentiation through the activation of protein kinase A (PKA) and the subsequent phosphorylation of cAMP response element binding protein (CREB).

Evidence also implicates protein kinase C (PKC) as a potential regulator of chondrogenic differentiation. For example, endogenous PKC activity increases during chondrogenesis *in vitro* (Sonn and Solursh, 1993). Moreover, inhibition or downregulation of intracellular PKC not only blocks the differentiation of prechondrogenic mesenchyme into chondrocytes (Choi et al., 1995), but also causes the dedifferentiation of fully differentiated chondrocytes (Bouakka et al., 1988).

Members of the TGF- β family have also been implicated as important regulators of skeletogenesis, perhaps initiating prechondrogenic mesenchyme cell aggregation. When injected subperiosteally, TGF- β 's stimulate the formation of ectopic cartilage *in vivo* (Joyce et al., 1990). Moreover, exogenous TGF- β ₁ promotes *in vitro* cartilage

differentiation by fibroblastic cells isolated from fetal rat muscle explants (Seyedin et al., 1985, 1986, 1987), and induces chondrogenic differentiation by embryonic chick limb mesenchyme cells plated at subconfluent cell densities that do not favor spontaneous chondrogenesis (Kulyk et al., 1989b). This stimulatory effect of TGF- β on prechondrogenic mesenchyme cell condensation and subsequent cartilage differentiation is associated with an enhancement of fibronectin mRNA accumulation (Leonard et al., 1991), and of NCAM expression (Roubin et al., 1990; Jiang et al., 1993; Widelitz et al., 1993), extracellular matrix macromolecules whose importance in the cellular aggregation phase have already been discussed. More recently, it has been demonstrated that mRNAs for endogenous TGF- β_2 , - β_3 , and - β_4 are expressed at their highest levels prior to or during the cellular aggregation phase of chondrogenesis in cultured chick limb mesenchyme cells *in vitro* (Roark and Greer, 1994).

Members of the BMP family of growth factors and the closely-related GDFs (growth and differentiation factors) are also amongst the molecules that have been implicated in the regulation of the critical cell condensation phase of chondrogenesis. The BMPs are a family of structurally-related secreted signaling molecules that form a subset of the TGF- β gene superfamily (Kingsley, 1994; Massague et al., 1994). They were originally isolated as factors capable of inducing ectopic bone formation when implanted subcutaneously or in muscle (Urist, 1965; Wozney et al., 1988). *In vivo*, various BMPs, including BMP-2 through BMP-7, have been shown to be localized to presumptive skeletal areas before the onset of condensation (Lyons et al., 1989; Lyons et al., 1990; Jones et al., 1991; Wozney et al., 1993; Vukicevic et al., 1994; Lyons et al., 1995). *In vitro* analyses with primary cell cultures and cell lines have further demonstrated that many of the BMPs induce cellular condensation and subsequent chondrogenic differentiation (Carrington et al., 1991; Chen et al., 1991; Roark and Greer, 1994; Rosen et al., 1994; Reddi, 1998; Shukumani et al., 1998; Stott et al., 1999), perhaps via cell adhesion molecules, such as N-cadherin, that are crucial to condensation (Haas and Tuan, 1999). Interestingly, BMPs applied exogenously to developing chick limb buds *in vivo* activate the expression of *Sox-9* (Healy et al., 1999), a gene encoding a transcription factor that has been shown to be required for the onset of cartilage-characteristic type II collagen gene expression (Lefebvre and de Crombrughe, 1998; Bi and de Crombrughe, 1999).

Further evidence that BMPs may play an important role in the initiation of chondrogenesis has come from the characterization of various null mutations in mouse BMP and BMP-related genes. For example, the mouse *short ear* mutation has been shown to result from an absence of BMP-5 protein (Kingsley et al., 1992). In *se/se*

mice, the affected axial skeletal structures exhibit reduced or absent mesenchymal cell condensations, leading to the subsequent skeletal abnormalities found at these sites (King et al., 1994). More recently, the mouse *brachypod* phenotype has been shown to arise from a defect in the GDF-5 locus, which prevents GDF-5 protein synthesis at specific sites that normally undergo chondrogenesis in the appendicular skeleton (Chang et al., 1994; Storm et al., 1994). As with *se/se*, the absence of GDF-5 protein results in the loss or malformation of specific skeletal elements. Interestingly, *in vitro* studies using tissues from *brachypod* mouse embryos have demonstrated that the mesenchymal cells present in these mice can be returned to normal function in the presence of inducing factors from wild-type littermates (Owens and Solursh, 1982). Following the chondrogenic differentiation of mesenchyme cell aggregates, several members of the BMP family continue to be expressed in the perichondrium (King et al., 1996), a sheath of cells that surrounds the differentiating cartilage element and provides a source of cells for both growth and repair of skeletal structures. It has been suggested that differential activity of the perichondrium may help control changes in the size and shape of skeletal structures (Thorogood, 1983). Thus, BMPs may play a role not only in initiating mesenchyme cell condensation and subsequent cartilage differentiation, but also in defining the size and shape of individual skeletal elements through the local regulation of the patterning of mesenchyme cell condensations.

BMPs signal by interacting with two classes of transmembrane ser/thr kinases known as type I and type II receptors. Ligand binding to both receptors results in the phosphorylation of the type I receptor by the type II receptor. The type I receptor then relays the signal by phosphorylating intracellular targets such as members of the Smad family of effector proteins (reviewed in Massague, 1996). Signaling through the various type I receptors by different TGF- β /BMP-family ligands may result in distinct intracellular responses (Massague, 1996). In other cases, however, qualitatively similar intracellular effects may be generated in response to closely-related BMP signals interacting with a shared group of receptors. Indeed, multiple BMPs and their respective receptors were found to be co-expressed spatially and temporally within the developing limb bud mesenchyme (Kawakami et al., 1996; Zou et al., 1997). To date, two type I BMP receptors, BMPR-IA (BRK-1) and BMPR-IB (BRK-2), and one type II receptor, BMPR-II, have been identified in vertebrates (Koenig et al., 1994; ten Dijke et al., 1994; Liu et al., 1995; Nohno et al., 1995; Rosenzweig et al., 1995; Massague, 1996). The two vertebrate type I BMPRs appear to play distinct roles in chondrogenesis (Zou et al., 1997). BMPR-IB is expressed in mesenchyme cell condensations immediately prior to overt cartilage differentiation, but it is not

detectable in early limb mesenchyme (Kawakami et al., 1996; Zou et al., 1997). Infection of chick limb buds with constitutively active BMPR-IB results in an expansion of the chondrogenic region *in vivo* and precociously stimulates chondrogenesis by limb mesenchyme cells *in vitro*. Moreover, ectopic expression of dominant-negative BMPR-IB disrupts cartilage formation *in vivo*, resulting in the loss of distal phalanges, and completely blocks chondrogenesis *in vitro*. These results suggest that BMPR-IB activity is essential for cartilage formation to occur both *in vivo* and *in vitro* (Kawakami et al., 1996; Zou et al., 1997). In contrast, BMPR-IA is expressed throughout early limb mesenchyme, although it subsequently becomes largely localized to prehypertrophic chondrocytes (Zou et al., 1997). Misexpression of constitutively active BMPR-IA results in a delay of terminal chondrocyte differentiation, suggesting that this type I receptor may play a role in the regulation of later stages of chondrogenic differentiation (Zou et al., 1997). Both BMPR-IA and BMPR-IB have also been implicated as potential regulators of programmed cell death in the developing limb bud, although their role in this process is presently less clear (Kawakami et al., 1996; Zou et al., 1997).

Exposure of responsive cells to growth factors has been shown to result in the rapid and transient activation of a set of immediate-early genes (Herschman, 1991; Lau and Nathans, 1991; Williams et al., 1992). These immediate-early genes encode proteins that are believed to mediate the biological responses of recipient cells to the growth factors. *In vivo*, the expression of one such gene and its secreted protein product, *Cyr61*, is transiently induced in chondrogenic mesenchyme cells of both mesodermal and neural crest origins (O'Brien and Lau, 1992) in response to stimulation by certain growth factors, such as those of the TGF- β and FGF families (O'Brien et al., 1990; Brunner et al., 1991; Yang and Lau, 1991). Functional evidence to support the suggestion that *Cyr61* may play a regulatory role in chondrogenic differentiation comes from *in vitro* studies demonstrating that exogenous *Cyr61* promotes chondrogenic differentiation in both high- and low-density cultures of prechondrogenic limb mesenchyme (Wong et al., 1997). In contrast, blocking *Cyr61* with specific antibodies has been shown to inhibit chondrogenesis (Wong et al., 1997).

1.5.5 Role of Transcription Factors in Regulating Cellular Condensation

In the past few years, considerable progress has also been made toward identifying nuclear factors that might regulate the expression of structural genes involved in prechondrogenic condensation and subsequent chondrocyte differentiation.

As discussed earlier, *Hox* genes are believed to play an important role in mediating the effects of early patterning signals. Analysis of skeletal defects produced by gain or loss of *Hox* gene function in the developing limb, however, has suggested that certain *Hox* genes may also play a more specific role in regulating mesenchyme cell condensation and chondrogenic differentiation (Morgan and Tabin, 1994; Yokouchi et al., 1995; Capecchi, 1996; Goff and Tabin, 1997). For example, *Hox A-13/Hox D-13* compound mutants exhibit an almost complete lack of chondrogenesis in the autopod (hand/footplate) of the developing limb (Dolle et al., 1993; Fromental-Ramain et al., 1996). *In vitro* analyses lend further support to this conclusion. *Hox D* antisense oligonucleotides have been shown to partially inhibit chondrogenesis in cultures of limb mesenchyme (Jung and Tsonis, 1998). The fact that complete inhibition of chondrogenesis was not observed may be explained by the existence of paralogous *Hox* genes on other *Hox* gene clusters that have identical boundaries of expression in chondrogenic tissues and that may functionally compensate for each other to some extent. Indeed, the *Hox* gene network and its overlapping, combinatorial domains of expression (Kessel and Gruss, 1990; Hunt and Krumlauf, 1991) is extremely complex, making a simple interpretation of these genes' functions in skeletogenesis difficult. Regardless, it does appear that various *Hox* genes are important in chondrogenic differentiation.

Members of the *Ets* superfamily of transcription factors (Thompson et al., 1991; Janknecht and Nordheim, 1992; reviewed in MacLeod et al., 1992) have also been implicated as potential regulators of embryonic cartilage formation. At mid-gestation, *Ets-2* RNA expression is localized to the developing limb buds and distal portion of the tail of the mouse embryo. Later, it becomes highly expressed in developing bone, as well as in several other tissues (Maroulakou et al., 1994). *Erg* is another member of the *Ets* family of transcription factors that has been shown to be widely expressed in mesodermal- and neural crest-derived tissues during early chick embryonic development. *Erg* subsequently becomes highly expressed in regions of precartilaginous condensations (Dhordain et al., 1995).

To date, however, only a single transcription factor has convincingly been shown to be required for the initiation of cartilage differentiation during embryonic development. *Sox-9* is a member of the *Sox* family of transcription factors which bear structural homology to the product of the SRY (sex-determining region of Y chromosome) gene by virtue of a conserved HMG-box DNA binding domain (Lefebvre and de Crombrughe, 1998; Wegner, 1999). Mutations in this gene in humans cause campomelic dysplasia, a severe dwarfism syndrome that affects all cartilage-derived

structures (Wagner et al., 1994). *Sox-9* mRNA is expressed in prechondrogenic mesenchyme both *in vivo* and *in vitro* (Wright et al., 1995; Ng et al., 1997; Zhao et al., 1997; Healy et al., 1999; Kulyk et al., 2000). Overexpression of *Sox-9* gene transcripts can induce ectopic cartilage formation *in vivo* (Healy et al., 1999). In contrast, prechondrogenic mesenchyme cells bearing a homozygous deletion of the *Sox-9* gene are unable to participate in cartilage formation in chimaeric mouse embryos and do not express cartilage-specific genes (Bi and de Crombrughe, 1999). In micromass cultures, *Sox-9* mRNA accumulates rapidly during the condensation phase of chondrogenesis, prior to the upregulation of type II collagen gene expression (Kulyk et al., 2000). A temporal precedence of *Sox-9* gene expression relative to type II collagen gene expression has also been demonstrated at sites of incipient cartilage formation *in vivo* (Ng et al., 1997; Zhao et al., 1997). The *Sox-9* protein activates type II collagen gene expression in prechondrogenic mesenchyme through its binding to an enhancer sequence within the Col2A1 first intron (Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997). Accordingly, there is a strong correlation between *Sox-9* and Col2A1 expression in chondrogenic mesenchyme during embryogenesis *in vivo* (Ng et al., 1997; Zhao et al., 1997). *Sox-9* also binds to an enhancer element in the gene encoding the $\alpha 2$ polypeptide chain of type XI collagen (Bridgewater et al., 1998), and may also regulate the transcription of other chondrocyte-specific genes (Xie et al., 1999), including adhesion molecules that promote prechondrogenic mesenchyme cell condensation (Healy et al., 1999). Together, these studies suggest that *Sox-9* may function as a "master switch" controlling chondrocyte differentiation.

1.6 Negative Modulators of Chondrogenesis

As previously discussed, ectodermal signals appear to play a crucial role in promoting the outgrowth and patterning of limb and facial primordia, and in regulating the sequential appearance of cartilage within these structures. The ectoderm also appears to suppress the chondrogenic potential of mesenchyme at the periphery, and thereby limits cartilage formation to its proper site at the core. Several lines of evidence support this conclusion. In cultures of both limb bud mesenchyme and facial mesenchyme, the presence of ectoderm inhibits chondrogenesis as evidenced by the lack of Alcian blue-stainable cartilage matrix (Solursh et al., 1981b; Wedden et al., 1986). Moreover, peripheral limb mesenchyme cells destined to form muscle and connective tissues *in vivo* readily form cartilage nodules when cultured in the absence of ectoderm (Zwilling, 1966; Solursh et al., 1981a). The interdigital region, which normally recedes and undergoes programmed cell death, can also undergo

chondrogenesis and form extra digits upon AER removal (Hurle and Ganan, 1987; Lee et al., 1994). It has been suggested that the ectoderm may exert such an inhibitory influence over subjacent mesenchyme by altering the peripheral extracellular matrix and establishing a non-chondrogenic region. Indeed, it has been demonstrated that the extracellular matrices of the prechondrogenic core and the peripheral region are morphologically distinct (Dessau et al., 1980; Singley and Solursh, 1981; Solursh et al., 1981b; Solursh et al., 1984).

Alternatively, the ectoderm may release diffusible "antichondrogenic" signaling molecules, and so prevent cartilage formation in the periphery of the limb. For example, several members of the Wnt gene family of secreted signaling factors have been implicated as regulators of skeletogenesis and pattern in the developing limb (Nusse and Varmus, 1992; Dealy et al., 1993; Parr and McMahon, 1994). More recently, it has been shown that ectopic expression of Wnt-1 in developing chick limb buds results in skeletal malformations that are consistent with the possibility that Wnt signaling molecules may inhibit or delay chondrogenesis (Rudnicki and Brown, 1997). Indeed, further analyses have demonstrated that expression of Wnt-1 in micromass cultures of limb mesenchyme dramatically suppresses chondrogenic differentiation. Wnt-7A, a gene that is endogenously expressed in the dorsal ectoderm of the limb (Dealy et al., 1993; Akita et al., 1996), has a similar inhibitory effect (Rudnicki and Brown, 1997; Stott et al., 1999). Together, these results suggest that Wnt signaling molecules may act to influence skeletal morphogenesis through the regulation of cartilage formation.

Retinoic acid (RA) is required for normal skeletal patterning in the developing limb (Noji et al., 1991; Wanek et al., 1991; Riddle et al., 1993; Helms et al., 1996; Stratford et al., 1996). Considerable evidence suggests, however, that RA may play an additional role in regulating chondrocyte differentiation. Indeed, RA has been shown to promote a dose-dependent inhibition of cartilage matrix formation and cartilage-specific gene expression in micromass cultures of limb mesenchyme (Hassell et al., 1978; Lewis et al., 1978; Horton et al., 1987; Paulsen et al., 1988). Moreover, mouse embryos exposed to high concentrations of RA during mesenchymal cell condensation and cartilage differentiation *in vivo* exhibit a variety of skeletal malformations, including the truncation of skeletal elements or, in more severe cases, the deletion of entire limb elements (reviewed in Underhill and Weston, 1998). The inhibitory effect of RA on chondrogenesis is believed to be mediated by nuclear hormone receptors of the retinoic acid receptor (RAR) and retinoid X receptor (RXR) subfamilies (each of which is comprised of 3 members, α , β and γ) that act as ligand-inducible transcription factors

(Underhill and Weston, 1998). Evidence suggests that members of the RAR/RXR families may play distinct roles in limb development and skeletogenesis. For example, both RAR α and RAR γ are initially expressed throughout prechondrogenic limb mesenchyme *in vitro* and *in vivo*. However, as mesenchyme cells aggregate and initiate overt cartilage differentiation, only RAR γ continues to be expressed at high levels. Downregulation of RAR α expression appears to be necessary to allow differentiation to occur.

The ERK Mitogen-Activated Protein (MAP) kinase has also been implicated as a negative regulator of cartilage differentiation. Inhibition of Erk-1 with the pharmacological agent, PD98059, enhances chondrogenesis in cultures of prechondrogenic limb mesenchyme, whereas inhibition of cartilage differentiation by phorbol esters leads to a rise in endogenous Erk-1 activity (Chang et al., 1998).

1.7 Advantages to an *In Vitro* Analysis of Chondrogenic Differentiation by Embryonic Limb Mesenchyme

In vivo skeletogenesis involves complex epithelial-mesenchymal, cell-cell and cell-matrix interactions between multiple cell populations of different embryological origins. Thus, a simplified *in vitro* model has been developed to facilitate the experimental analysis of chondrogenic differentiation (reviewed in Daniels et al., 1996). Indeed, much of our current knowledge of the molecular events that regulate chondrocyte differentiation has come from *in vitro* studies of cartilage formation in high-density micromass cultures of embryonic limb mesenchyme cells. The success of the micromass culture system lies in its ability to mimic the events occurring during *in vivo* chondrocyte differentiation. Thus, progression of cells through the chondrogenic pathway can be followed *in vitro* by analysis of specific cytodifferentiation markers. The micromass culture system has also been shown to be extremely versatile and readily manipulable. For example, culture conditions affecting the ability of limb mesenchyme cells to spontaneously differentiate, such as cell density and serum concentrations, can easily be varied. Moreover, pharmacological agents, growth factors, antisense oligonucleotides, etc. can be added exogenously to cultures, and as such, provides a convenient means of analyzing the effect of specific factors on chondrogenic differentiation, or for identifying teratogens capable of perturbing skeletal development (Hassell and Horgan, 1982; Paulsen et al., 1988).

Most frequently, micromass cultures are prepared from mesenchyme cells of whole stage 23 - 25 chick embryo wing buds (Ahrens et al., 1977), although they may also be prepared from mouse and rat embryo limb buds of equivalent morphological

stages (Langille, 1994; Wong et al., 1997). Briefly, wing buds are isolated from stage 23/24 chick embryos, and dissociated into a single cell suspension ($1 - 2 \times 10^7$ cells/ml tissue culture medium) which is subsequently spotted in 10 - 20 μ l drops onto tissue culture plates. Once attached to the substratum, the limb mesenchyme cells are flooded with tissue culture medium containing the agent to be tested, and incubated at 37°C in a 5% CO₂ incubator for various lengths of time.

Over a 3-day period of culture, the mesenchyme cells plated at superconfluent density progress through the various stages of chondrocyte differentiation, and closely parallel the chondrogenic events taking place *in vivo* (Archer et al., 1982). During the first day of incubation, the limb mesenchyme cells form prechondrogenic aggregates. During the second and third days of incubation, the limb mesenchyme cells within these nodular aggregates begin to express high levels of type II collagen and aggrecan gene transcripts, and progressively secrete an Alcian blue-positive cartilage matrix. Levels of type II collagen and aggrecan proteins and their respective gene transcripts provide convenient markers for quantifying the extent of cartilage differentiation (Kravis and Upholt, 1985; Mallein-Gerin et al., 1988; Swalla et al., 1988; Stirpe and Goetinck, 1989).

Detailed analysis of the molecular events accompanying chondrocyte differentiation in micromass cultures prepared from whole limb mesenchyme is complicated by the fact that the mesenchymal cell population contains myogenic progenitor cells in addition to prechondrogenic cells. To circumvent this problem, micromass cultures can be prepared from "subridge" or "progress zone" mesenchyme isolated from the distal tips of stage 24/25 chick embryo wing buds (Gay and Kosher, 1984). Cells isolated from this region represent a more homogeneous population of prechondrogenic mesenchyme since myoblast progenitors are restricted to more proximal limb regions at this stage (Newman et al., 1981; Gay and Kosher, 1984). Moreover, the subridge mesenchyme has not yet initiated chondrogenic differentiation *in situ* due to its proximity to the AER. Micromass cultures of stage 24/25 distal tip limb mesenchyme exhibit a more rapid accumulation of type II collagen and aggrecan RNA sequences, and a more uniform elaboration of Alcian blue-positive cartilage matrix, relative to micromass cultures prepared from whole wing bud mesenchyme. By three days of incubation, the cultures form a virtually continuous sheet of cartilage tissue (Gay and Kosher, 1984). This distal tip culture system has been extremely well-characterized with respect to the expression of cartilage-specific genes, including types II and IX collagen (Kosher et al., 1986a; Kulyk et al., 1991), aggrecan (Kosher et al., 1986b), fibronectin (Kulyk et al., 1989a), and *Sox-9* (Kulyk et al., 2000).

The capacity of embryonic limb mesenchyme cells for spontaneous cartilage formation in micromass cultures is dependent on a superconfluent plating density (Ahrens et al., 1977; Umansky, 1996). As previously reported (Kulyk et al., 1989b), when limb mesenchyme cells are plated at a reduced density, the cells undergo relatively little spontaneous cartilage differentiation. Therefore, such low-density cultures of prechondrogenic limb mesenchyme cells have been used to identify factors that are capable of inducing chondrogenic differentiation where it otherwise doesn't occur (Kulyk et al., 1989b; Kulyk, 1991).

1.8 Cartilage Maturation and Hypertrophy

Hyaline cartilage is a permanent tissue only in certain areas of the body (e.g., articular cartilage of joint surfaces, nasal septum cartilage). Most chondrocytes, however, undergo further major phenotypic changes during late embryogenesis and early postnatal life, as they hypertrophy and secrete a different extracellular matrix during the endochondral ossification process. As will be discussed, perhaps the most dramatic change observed in the extracellular matrix of hypertrophic cartilage is the appearance of type X collagen, prior to the mineralization of the extracellular matrix (Gibson and Flint, 1985; Schmid and Linsenmayer, 1985a,b).

The transition from cartilage to bone during skeletal development is a multistep process involving cartilage synthesis, hypertrophy, calcification, degradation, and replacement of cartilage by bone and marrow, as most clearly defined in studies of the growth plates of developing long bones (reviewed in Poole, 1997). Within each growth plate, several distinct histological regions are observed that contain chondrocytes at sequential stages of differentiation. In the reserve zone, cells exhibit little or no cell division, whereas in the proliferative zone, cells rapidly divide to give rise to columns of flattened chondrocytes which elaborate a characteristic hyaline cartilage matrix that is rich in type II collagen and aggrecan. These cells then begin to swell into large, round hypertrophic chondrocytes within the zone of maturation, and finally enter the hypertrophic zone. As chondrocytes undergo hypertrophy, type X collagen synthesis is initiated, type II collagen production ceases, and alkaline phosphatase levels rise dramatically. Thus, cartilage which is undergoing endochondral ossification can be distinguished by the presence of hypertrophic chondrocytes and their unique collagen type X product (Gibson and Flint, 1985; Schmid and Linsenmayer, 1985a,b).

Mineralization or calcification of cartilage takes place in the hypertrophic zone, beginning in discrete focal sites between collagen fibrils, and eventually progressing throughout the longitudinal septa of the cartilage matrix. The transverse septa do not

calcify, nor do the pericellular zones immediately surrounding the chondrocytes. At this time, capillary sprouts erode the last transverse septa separating the hypertrophic zone from the metaphysis. Eventually, vessels break through into the lacunae containing the remnants of hypertrophic cells. Many of the mineralized longitudinal septa are eroded by chondroclasts. Those that remain are now called trabeculae and act as calcified cartilaginous scaffolds onto which osteoblasts settle and secrete osteoid which subsequently calcifies, giving rise to woven bone overlying the calcified cartilaginous trabeculae. Eventually, this woven bone and calcified trabeculae are resorbed by osteoclasts and replaced by a mature lamellar, or cancellous, bone.

As mineralization proceeds, expression of marker genes of the osteoblastic phenotype is initiated. For example, synthesis of both osteocalcin and osteopontin has been observed in chick embryo vertebral chondrocyte cultures that have been incubated under conditions that promote differentiation toward hypertrophy and extracellular matrix mineralization (Lian et al., 1993).

1.9 Biological Effects of Alcohol

1.9.1 Fetal Alcohol-Related Birth Defects

Ethanol, like many recreational and prescription drugs, has been shown to cause an increased incidence of congenital birth defects in humans. However, as a widely available, socially acceptable, legal intoxicating substance, alcohol is currently the most commonly used teratogen in humans. Fetal Alcohol Syndrome (FAS), with a worldwide incidence of approximately 1/1000 live births, is a complex of congenital malformations resulting from chronic exposure to a high level of alcohol during gestation (Abel, 1984; Webster, 1989). FAS has three diagnostic criteria: (1) growth retardation, both pre- and postnatally; (2) abnormalities of the central nervous system (CNS) that include mild to moderate mental retardation, microcephaly at birth, and irritability in infancy; (3) characteristic facial dysmorphologies, including short palpebral fissures (width of the visible eye), a short upturned nose with a low bridge, a long distance between the nose and the vermilion of the upper lip, an absent or indistinct philtrum (vertical ridge between the nose and upper lip), thin upper lip vermilion, a flat midface, and retrognathia (small lower jaw). In addition to being responsible for the specific group of malformations that define full-blown FAS, intrauterine alcohol exposure has been linked to a wide variety of other anomalies of embryonic organ primordia, including skeletal, cardiac, CNS, and urogenital structures (Herrmann et al., 1980; Streissguth et al., 1980; Sulik et al., 1981; Webster et al., 1983;

Abel, 1984; Pauli and Feldman, 1986; Webster, 1989; Schenker et al., 1990; Webster and Ritchie, 1991; Becker et al., 1996; Johnson et al., 1996). These malformations may occur in isolation or in various combinations. Thus, it is now appreciated that FAS represents only one pattern of possible alcohol damage. As such, the term Fetal Alcohol-related Effects (FAE) is more inclusive when describing the congenital anomalies induced by prenatal alcohol exposure (Abel, 1984). Indeed, the worldwide incidence of FAE is believed to be as high as 3-4/1000 live births, or approximately 5% of all congenital malformations in Western urban populations (Sokol et al., 1980; Abel, 1984). Children with FAS or FAE, however, only represent the surviving embryos. There is also evidence that alcohol abuse during pregnancy results in an increased incidence of spontaneous abortion (Kline et al., 1980).

1.9.2 Animal Models of Fetal Alcohol Effects

Studies of ethanol teratogenicity in laboratory animals have typically involved the administration of a single large dose of ethanol to pregnant animals during critical periods of intense embryonic cell proliferation and differentiation. This type of acute alcohol exposure does not duplicate the pattern of human exposure since most, if not all, children diagnosed with FAS/FAE are born to women who are chronic alcoholics (Abel, 1999). However, this type of investigation does reveal the most sensitive stages of embryonic development to high doses of the test agent, and is the most convenient way to demonstrate the range of possible malformations inducible by the agent and their dose dependence.

In general, not all stages of embryonic development are equally sensitive to teratogens (Webster, 1989). It has been demonstrated, in both animal and human studies, that exposure to alcohol during the preorganogenic period generally causes spontaneous abortion, rather than malformation (Checiu and Sandor, 1981). In contrast, embryos acutely exposed to high levels of alcohol during the organogenic period (7 - 12 days of murine gestation; comparable to 3 - 8 weeks of human development) exhibit an increased incidence of congenital malformations (Abel, 1984; Sulik et al., 1988; Webster and Ritchie, 1991). The type of malformation induced is dependent upon the specific stage of development at the time of exposure. For example, mouse embryos exposed to teratogenic doses of ethanol (0.03 ml 25% ethanol/g body weight, injected intraperitoneally, which is roughly equivalent to peak maternal blood alcohol levels of between 400 - 800 mg/dl) on day 7 of gestation exhibited a wide range of malformations of the face and CNS (Webster et al., 1983). The rostral part of the CNS appears to be particularly vulnerable since some embryos were missing the entire

forebrain and had virtually no face. Most embryos were affected less severely, exhibiting facial features very similar to those of FAS in humans as described above (Sulik et al., 1981; Sulik and Johnston, 1983; Webster et al., 1983). In addition, similar ethanol exposure on day 7 of gestation interrupted the process of neural tube closure (Clarren, 1979; Majewski, 1981). This resulted in exencephaly, in which the rostral part of the neural tube remains open, and corresponds to the lethal human malformation, anencephaly. Alcohol exposure on day 8 of gestation induced facial abnormalities such as cleft palate, as well as defects of the heart and thymus (Webster et al., 1983; Daft et al., 1986). Since cartilage, bone and connective tissues of the face, and connective tissues of the heart and thymus are neural crest-derivatives (LeLievre, 1978; Kirby et al., 1983; Noden, 1983; Bockman and Kirby, 1984; Couly et al., 1993), it has been suggested that neural crest may be a particularly sensitive target of ethanol teratogenicity, and that these malformations may be due to the effect of ethanol on neural crest cell migration, development, and viability (Webster et al., 1983; Hassler and Moran, 1986; Sulik et al., 1986; Cartwright and Smith, 1995a,b; Rovasio and Battiato, 1995; Chen and Sulik, 1996). Alcohol exposure on days 9-12 of gestation resulted in a high incidence of limb defects that have also been described in children with FAS/FAE, including a reduction in the size of digits, the fusion of digits (syndactyly), extra digits (polydactyly), and the complete lack of digits (ectrodactyly). Less frequently, more proximal limb elements may also be affected (Herrmann et al., 1980; Webster et al., 1983; Pauli and Feldman, 1986; Kotch et al., 1992; Zajac and Abel, 1992). Cleft palate, and heart and renal anomalies were also prevalent. The remainder of gestation is known as the fetal period, and is a time of intense growth and CNS development. Although alcohol administered during this phase does not typically produce structural malformations, it can promote growth retardation, mental retardation, and behavioral abnormalities (Webster, 1989).

The congenital malformations observed following chronic alcohol exposure are similar to those seen in acute administrations studies, provided that the maternal blood alcohol levels intermittently reaches 200-300 mg/dl or higher (i.e., greater than 40 mM) (Webster, 1989). For example, miniature swine consuming 20% alcohol in their drinking water for an extended period prior to and during pregnancy gave birth to severely growth-retarded litters with a high incidence of congenital malformations that included microphthalmia, micrognathia, cleft palate, microcephaly, and abnormalities of the extremities and genitalia. Peak maternal blood alcohol levels up to 373 mg/dl were measured in this study (Dexter et al., 1980). In another study, the pups of pregnant dogs exposed daily to high levels of alcohol exhibited tail defects and renal agenesis.

These effects were associated with daily maternal blood alcohol levels of approximately 200 mg/dl. There were no viable offspring when daily blood alcohol levels reached 250-350 mg/dl (Ellis and Pick, 1980). Pregnant mice given liquid diets and having blood alcohol levels in excess of 261 mg/dl produced growth-retarded fetuses that exhibited a wide range of malformations of the heart, limbs, eyes, and genitourinary system (Chernoff, 1977).

The frequency with which these high levels of alcohol are attained in the general human population is currently unknown. Blood alcohol levels of 300 mg/dl will produce a stuporous condition in the average person. However, chronic alcoholics often exhibit little clinical evidence of intoxication at these levels, and have been reported to maintain consciousness after reaching levels as high as 780 mg/dl (Lindblad and Olsson, 1976; Davis and Lipson, 1986). Indeed, there have been few, if any, instances of a child with FAS/FAE being born to a woman who was not a chronic alcoholic or binge drinker (Abel, 1999).

1.9.3 Alcohol Metabolism

In mammals, ethanol metabolism occurs mainly in the liver, via a 2-step pathway (reviewed in Crow and Hardman, 1989). Ethanol is first oxidized to acetaldehyde, a reaction catalyzed by alcohol dehydrogenase (ADH). Subsequently, aldehyde dehydrogenase (ALDH) rapidly oxidizes acetaldehyde to acetate which is then exported from the liver to be further metabolized by peripheral tissues. Within a few minutes of ethanol reaching the liver, the ratio of free $[NAD^+]/[NADH]$ in the cytoplasmic and mitochondrial compartments is reduced. This occurs because both ADH and ALDH-catalyzed reactions require NAD^+ , and produce $NADH$. This $NADH$ must be reoxidized for oxidative metabolism to continue. Therefore, the immediate metabolic effects of acute ethanol exposure on the liver are production of acetaldehyde and acetate, a decrease in free cytosolic and mitochondrial $[NAD^+]/[NADH]$ ratios, and altered oxygen utilization. The metabolism of an acute dose of ethanol by the liver leads to secondary changes in many of the major metabolic pathways, such as those involved in glucose and lipid metabolism.

The ADH family consists of numerous enzymes that are able to catalyze the oxidation of a wide variety of alcohols to their corresponding aldehydes. Several distinct classes of vertebrate ADH have been described, all of which are cytosolic and zinc-dependent, but differ in substrate specificities and gene expression patterns (Duester, 1996). Three forms that are highly conserved among vertebrates are class I ADH, class III ADH, and class IV ADH. Evidence indicates that these three

ADHs are able to utilize a wide variety of alcohol and aldehyde substrates *in vitro*, ranging from ethanol to formaldehyde to retinol (Allali-Hassani et al., 1998; Han et al., 1998; Kedishvili et al., 1998). Although primarily defined as liver ADH, class I ADHs have also been detected in several other adult tissues (Smith et al., 1972; Goedde et al., 1979). More recently, it has been demonstrated that murine class I ADH (Adh-1) is also expressed in several embryonic tissues and in discrete mesenchymal regions (Vonesch et al., 1994; Ang et al., 1996). To date, however, only class IV ADH has been detected in craniofacial and limb bud mesenchyme (Ang et al., 1996). The principal function of class IV ADH is the metabolic conversion of endogenous retinol (vitamin A) to retinoic acid. Ethanol is a much poorer substrate for class IV ADH, but at teratogenic doses it has been demonstrated to compete with retinol for access to the enzyme (Duester, 1991).

The question has arisen as to whether ethanol damage to the embryo is due to the direct toxic effects of ethanol, its acetaldehyde metabolite, or a combination of the two chemicals. Indeed, both ethanol and acetaldehyde have been shown to cause growth retardation and congenital malformations in developing mouse embryos (Dreosti et al., 1981; Webster et al., 1983; Blakely and Scott, 1984). However, animals treated with both alcohol and ADH inhibitors, which should elevate acetaldehyde levels, do not exhibit a significantly greater incidence of malformations than animals exposed to alcohol alone (Webster et al., 1983). In contrast, pyrazole, an ADH inhibitor, increases the embryotoxicity and teratogenicity of ethanol (Ukita et al., 1993). These results suggest that ethanol is the proximal teratogen of FAS/FAE, although the contribution of acetaldehyde cannot be ruled out completely.

1.9.4 Effects of Alcohol on Embryonic and Cellular Metabolism

Despite an extensive literature documenting the deleterious effects of ethanol on vertebrate embryogenesis, the molecular mechanisms underlying its teratogenicity are currently unclear. Many investigators have attributed ethanol's pathogenicity to generalized cytotoxicity, or to disturbances of systemic physiological processes (Streissguth et al., 1980; Sulik et al., 1981; Webster, 1989; Schenker et al., 1990; Kotch and Sulik, 1992; Kotch et al., 1992; Zajac and Abel, 1992; Henderson et al., 1995; Shibley and Pennington, 1997).

At high concentrations, alcohol is cytotoxic, resulting in heightened cell death within developing organ primordia (Sulik et al., 1988; Webster, 1989; Kotch and Sulik, 1992). However, the mechanism(s) underlying ethanol-induced cell death is unclear. There are two broad mechanisms of cell death, necrosis and apoptosis, that are

distinguished by unique morphological and biochemical features (reviewed in White, 1996). In response to injury (e.g., organelle failure), cells swell, die and subsequently lyse. Apoptosis, on the other hand, is a cellular response to specific signals that activate a cascade of intracellular events, and leads to irreversible or programmed cell death. The latter is often used to eliminate unwanted cells during embryogenesis. For example, programmed cell death occurring in the anterior and posterior necrotic zones of the early limb bud, and subsequently in the interdigital region, is involved in sculpting and contouring the developing limb bud and digits (Saunders et al., 1962). Likewise, apoptosis eliminates cranial neural crest cells from specific hindbrain segments, thereby preventing them from contributing to craniofacial morphogenesis (Graham et al., 1994). It has been demonstrated that following administration of a teratogenic dose of ethanol, mouse embryos exhibit enlarged mitochondria and an increased number of cell inclusions which are indicative of necrotic cell death (Webster, 1989). However, more recent studies in a chick embryo model of FAS demonstrate that ethanol exposure causes apoptosis, not necrosis, of cranial neural crest (Cartwright et al., 1998). Although this ethanol-induced apoptosis occurs coincident with the normal deletion of unwanted cranial neural crest cells, ethanol treatment fails to activate two components of the endogenous apoptotic pathway, BMP-4 and *Msx-2* (Graham et al., 1994; Davidson, 1995). These results instead implicate activation of a distinct pathway of programmed cell death that subsequently converges with the endogenous pathway. Indeed, simultaneous activation of convergent apoptotic mechanisms has been demonstrated in ethanol-treated thymocytes (Ewald and Shao, 1993).

Ethanol's teratogenic effects have also been attributed to systemic disturbances of embryonic metabolism and/or placental function. Indeed, ethanol has been shown to alter fetal nutrient supplies via alterations in blood flow and placental/fetal perfusion (Hoyseth and Jones, 1989). Other molecular alterations known to result from fetal alcohol exposure include changes in the metabolism of minerals, amino acids, and other compounds. For example, alcohol has been shown to interfere with placental transport and fetal uptake of zinc (reviewed in Seyoum and Persaud, 1990). Even a mild deficiency of zinc has been observed to be teratogenic, resulting in fetal growth retardation and skeletal defects. Since zinc deficiency reduces ADH activity, it may be that the process of ethanol elimination is slowed down and results in higher circulating and tissue ethanol levels for a prolonged period. Ethanol teratogenicity has also been attributed to reduced levels of both glutathione, which is involved in hepatic detoxification, and the essential amino acid, methionine, which has been reported to reduce circulating acetaldehyde (Seyoum and Persaud, 1990). Further evidence to

support these reports comes from the observation that organic zinc salts and thiols increase the survival of mice poisoned with a lethal dose of ethanol (Seyoum and Persaud, 1990).

In addition to generalized disturbances of systemic physiological processes, ethanol has been shown to modulate a variety of crucial signal transduction pathways in cultured neural, hepatic and other cell types (Hoek and Rubin, 1990). Some of the diverse cellular effects of alcohols are associated with their ability to alter biological membrane fluidity and thereby influence an array of membrane-associated signaling processes (Taraschi and Rubin, 1985; Littleton, 1989). For example, ethanol exposure has been shown to modulate interactions between signaling ligands and their receptors (Henderson et al., 1989; Resnicoff et al., 1993; Tomono and Kiss, 1995). It also influences adenylate cyclase activity and cyclic AMP production (Weston and Greene, 1991; Nagy and DeSilva, 1992; Rabin et al., 1992), as well as arachidonate metabolism and prostaglandin synthesis (Szabo et al., 1992; Balsinde, 1993; Navamani et al., 1997). Still other membrane-associated effects of ethanol include phospholipid turnover and phosphoinositide metabolism (Rooney et al., 1989; Kiss and Garamszegi, 1993), G protein activation and intracellular protein kinase activities (Messing et al., 1991; DePetrillo and Liou, 1993; Slater et al., 1993; Reddy and Shukla, 1996), and the transport of Ca^{2+} and other ions through membrane channels (Littleton et al., 1991; Rout et al., 1997). Virtually all of these molecular mechanisms have been implicated at some level in the regulation of embryonic cartilage formation. Thus, there are a multiplicity of routes through which ethanol might conceivably influence chondrogenic differentiation by limb and facial mesenchyme. For example, the activation of membrane receptor kinases for growth factors of the FGF, TGF- β , PDGF, IGF and BMP families is believed to regulate the mitotic and differentiative activities of prechondrogenic mesenchyme cells during limb morphogenesis *in vivo* (Centrella et al., 1988; Kulyk et al., 1989b; Leonard et al., 1991; Tabin, 1991; Niswander and Martin, 1993a; Savage et al., 1993; Laufer et al., 1994; Dealy and Kosher, 1995). Elevated prostaglandin production (Chepenik et al., 1984; Biddulph et al., 1988; Capehart and Biddulph, 1991), and an ensuing rise in intracellular cyclic AMP (Elmer et al., 1981; Ho et al., 1982; Kosher and Walker, 1983) are associated with the critical period of prechondrogenic mesenchymal cell condensation that precedes overt chondrocyte differentiation in the limb. Modulations of protein kinase C activity (Garrison et al., 1987; Kulyk, 1991; Sonn and Solursh, 1993), and intracellular Ca^{2+} levels (Bouakka et al., 1988) in cultured limb mesenchyme cells have also been demonstrated to influence the extent of *in vitro* cartilage formation.

Alcohol treatment might also affect the expression of genes and secreted signaling molecules that regulate skeletal patterning and chondrogenic differentiation. For example, ethanol treatment not only inhibits the expression of *Msx-2* gene transcripts in cultured calvarial osteoblasts, but it also completely abolishes *Msx-2* expression in developing mouse embryos *in vivo* (Rifas et al., 1997). As such, it has been suggested that inhibition of *Msx-2* expression may be a mechanism through which ethanol exerts its pathogenic effects on vertebrate embryogenesis (Rifas et al., 1997). It has also been hypothesized that ethanol may perturb embryonic morphogenesis *in vivo* by competing with retinol (vitamin A) for ADH, an enzyme that is involved in the pathway of retinoic acid synthesis and in the metabolic conversion of alcohol to acetaldehyde (Duester, 1991; Deltour et al., 1996). Indeed, endogenous retinoids are believed to play crucial roles in the patterning of skeletal elements along the A-P axis of the developing limb bud, and exogenous retinoids have been shown to exert profound effects on limb cartilage differentiation *in vitro* (Hassell and Horigan, 1982; Paulsen et al., 1988; Tabin, 1991; Underhill and Weston, 1998).

Recent investigations indicate that ethanol may also exert more selective influences on specific progenitor cell populations of embryonic organ primordia. For example, ethanol exposure has been shown to preferentially depress the viability and/or motility of cranial neural crest cells at the time they migrate from the converging neural folds into the developing facial primordia (Cartwright and Smith, 1995a,b; Rovasio and Battiato, 1995; Chen and Sulik, 1996). This may explain, at least in part, the high incidence of facial dysmorphologies associated with prenatal alcohol exposure in both humans and laboratory animals.

1.10 Experimental Objectives

The potential for ethanol to influence signaling pathways regulating chondrogenesis, together with the well-documented effects of ethanol on embryonic skeletogenesis, prompted us to examine whether ethanol exposure might directly influence the ability of prechondrogenic mesenchyme cells to initiate cartilage differentiation *in vitro*. Accordingly, my thesis work focused on characterizing the effects of alcohol exposure on chondrocyte differentiation in cultures of prechondrogenic mesenchyme cells isolated from the limb and facial primordia of the chicken embryo, and on investigating the molecular pathways that mediate ethanol's effects on *in vitro* chondrogenesis.

The specific aims of my project were:

- 1) to examine the effects of ethanol and other alcohols on levels of cartilage matrix formation in cultures of prechondrogenic mesenchyme cells isolated from the limb buds and facial primordia (maxillary, mandibular, frontonasal, and hyoid processes) of the chick embryo.
- 2) to examine the effects of alcohol exposure on the expression of genes for the principal cartilage matrix structural components, type II collagen and aggrecan, in cultures of limb and facial mesenchyme.
- 3) to examine whether ethanol treatment influences cellular proliferation in prechondrogenic limb mesenchyme cultures, and to determine whether ethanol's effects on *in vitro* chondrogenesis are dependent on altered cellular proliferation.
- 4) to determine whether ethanol's effects on matrix accumulation and cartilage-specific gene expression are restricted to chondrogenic *progenitor* cells, or whether ethanol treatment similarly affects the expression of cartilage-specific phenotypic markers in well-differentiated sternal chondrocytes.
- 5) to determine whether *in vitro* ethanol treatment alters the expression of several putative chondrogenic regulatory genes (*Sox-9*, *BMPR-IB*, *TGF- β* , *Ets-2*, and *Erg* mRNAs). In addition, an attempt was made to identify novel genes that are preferentially expressed during ethanol-induced chondrocyte differentiation.
- 6) to determine whether ethanol treatment of cultured prechondrogenic limb mesenchyme cells alters the expression of the *Msx-2* homeobox gene, which has been implicated in alcohol's teratogenic effects *in vivo*.
- 7) to examine whether exposure of prechondrogenic mesenchyme cells to ethanol induces a classic stress response, as characterized by rapid induction of Hsp genes.
- 8) to examine which intracellular signaling pathways might be required to mediate the stimulatory effects of ethanol on *in vitro* cartilage differentiation.

2. MATERIALS AND METHODS

2.1 Animals

Fertilized chicken eggs (Cornish Cross, White Leghorn, or Red Sussex) were obtained from Anstey Hatchery (Saskatoon, Saskatchewan), and maintained at 38°C - 39°C in a humidified, forced-air incubator until embryos reached the desired stage of development. At the desired time point, the eggs were cracked and the embryos staged according to the morphological criteria of Hamburger and Hamilton (1951), in order to select embryos of equivalent developmental stage. Typically 40 - 60 % of eggs contained embryos of the desired stage. All others were discarded.

2.2 Establishment of Dissociated Cell Cultures from Embryonic Chick Limb and Facial Prechondrogenic Mesenchyme and Differentiated Sternal Chondrocytes

2.2.1 Preparation of Cultures from Embryonic Chick Limb Prechondrogenic Mesenchymal Cells

For each experiment, wing buds were dissected from approximately 10 - 15 dozen stage 23/24 (4 days post-fertilization) or 15 - 30 dozen stage 24/25 (4.5 days post-fertilization) (Hamburger and Hamilton, 1951) chick embryos. Distal wing bud tips (subridge regions) were dissected from stage 24/25 wing buds as described by Gay and Kosher (1984), with the excised region extending 0.3 - 0.4 mm from the distal apex of the limb to the proximal cut edge. As previously detailed (Kulyk, 1991), ectoderm was stripped from the limb tissues following treatment with 0.8 unit/ml dispase. The isolated distal subridge or whole wing bud mesoderm tissues were then incubated for 20 min. in 0.25% trypsin and subsequently dissociated into a single cell suspension in F12/10% FBS medium (Ham's F12 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and antibiotics: 100 µg/ml kanamycin, 100 U/ml penicillin G, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B). Cell density was determined using a hemocytometer and adjusted to a final density of $1 - 2 \times 10^7$ cells/ml by addition of the required volume of F12/10% FBS medium. High-density micromass cultures were established by spotting 10 or 20 µl drops of cell suspension onto plastic tissue culture plates (Falcon or Nunclon 35 mm and 60 mm dishes; Nunclon 4-well

plates and 24-well plates) following standard procedures (Ahrens et al., 1977; Gay and Kosher, 1984). Low-density cultures of limb mesenchyme were established as previously described (Kulyk et al., 1989b) by spotting 10 or 20 μ l drops of cell suspension that had been further diluted to a final density of 0.5×10^7 cells/ml in F12 medium. All plates were incubated for 90 min. at 37°C in a 5% CO₂ incubator to permit cell attachment. High-density micromass cultures were then flooded with F12/10% FBS medium supplemented with 14 mM Hepes, pH 7.2 and any agents being tested. The time of initial feeding represented 0 hour time points. Low-density cultures were usually preincubated in F12/1% FBS medium for 6 - 12 hours before any treatments were performed. This maximized cell survival upon subsequent addition of medium containing a final concentration of 10% serum and any agent being tested.

For alcohol treatments as specified, the feeding medium was supplemented with reagent grade ethanol, methanol, 2-propanol, or tertiary butanol (2-methyl-2-propanol) to final concentrations of 0.1 - 4.0% (v/v). The medium of parallel control cultures was supplemented with an equivalent volume of sterile distilled water. In most experiments, the F12/10% FBS medium was additionally supplemented with 14 mM Hepes, and the cultures were tightly sealed with parafilm to minimize evaporative loss of alcohols during subsequent incubation. However, in initial experiments, the plates were left unsealed. (In preliminary trials, alcohol treatment effects were found to be qualitatively equivalent under sealed and unsealed incubation conditions; data not shown). Where specified, the feeding medium was supplemented with various protein kinase inhibitors, or cytosine arabinoside. In these experiments the medium of parallel control cultures was supplemented with an equivalent volume of sterile distilled water or DMSO, depending on which solvent the inhibitor was dissolved in. The cultures were then incubated for various lengths of time (0 hr - 72 hr; see individual experiments) at 37°C in a 5% CO₂/95% air atmosphere without subsequent medium change. At the end of the incubation period, cultures were processed for histochemical staining (described below in 2.3.1), measurement of matrix glycosaminoglycan synthesis (described below in 2.3.2 and 2.4), or analysis of cartilage-specific mRNA expression (described below in 2.6).

In experiments utilizing various protein kinase inhibitors, cultures were fed initially either with control medium, or with medium containing a specific pharmacological inhibitor. The concentration of inhibitor used was based on previous dose-response experiments conducted in our laboratory, on published K_i constants (inhibition constants) and IC₅₀ values (concentration required to produce 50% inhibition), and on published reports of the use of these inhibitors in other cell types.

Following a 12 hour preincubation with the inhibitor alone, the cultures were incubated for an additional 48 - 60 hours in the presence of both inhibitor and 1.5% ethanol, or inhibitor alone.

In experiments examining the effects of ethanol on cellular proliferation, high- and low-density cultures were incubated overnight in F12/10% FBS or F12/1% FBS, respectively. The following morning, cultures were fed with medium containing final concentrations of 10% FBS, 5 $\mu\text{Ci/ml}$ ^3H -thymidine, 1.5% ethanol, and/or 0.5 $\mu\text{g/ml}$ cytosine arabinoside.

2.2.2 Establishment of Cultures from Embryonic Chick Facial Mesenchyme Cells

Maxillary, mandibular, frontonasal, and hyoid facial processes were dissected from stage 24/25 chick embryos (Hamburger and Hamilton, 1951) as previously described (Wedden, 1986; Kulyk and Reichert, 1992). The isolated facial primordia were stripped of surface ectoderm following dispase treatment, incubated in 0.25% trypsin, and dissociated into a suspension of 2×10^7 cells/ml in DMEM:F12/10% FBS medium (a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12 medium containing 10% fetal bovine serum, 14 mM Hepes, pH 7.2, 2 mM glutamine, and antibiotics: 100 $\mu\text{g/ml}$ kanamycin, 100 U/ml penicillin G, 100 $\mu\text{g/ml}$ streptomycin, 0.25 $\mu\text{g/ml}$ amphotericin B) (Kulyk and Reichert, 1992). Micromass cultures of facial mesenchyme were established by spotting 10 μl drops of cell suspension (2×10^7 cells/ml) onto plastic tissue culture plates (35 mm Nunclon dishes; 4- or 24-well Nunclon plates). After incubation for 1.5 - 2.0 hours at $37^\circ\text{C}/5\% \text{CO}_2$ to permit cell attachment, the plates were flooded with DMEM:F12/10% FBS medium. For alcohol treatments, the medium was supplemented with reagent grade ethanol or tertiary butanol. Control cultures were supplemented with an equivalent volume of sterile distilled water. The plates were sealed with parafilm to minimize evaporation, and cultured for 3 days at 37°C in a $5\% \text{CO}_2$ incubator without medium change. At the end of the incubation period, cultures were processed for histochemical staining (described below in 2.3.1), measurement of matrix glycosaminoglycan synthesis (described below in 2.4), or analysis of cartilage-specific mRNA expression (described below in 2.6).

2.2.3 Establishment of Cultures from Embryonic Chick Sternal Chondrocytes

Sterna were dissected from 16 - 18 day old chicken embryos, and the perichondrium removed manually. The sterna were subsequently cut transversely into

1/3's, and the upper and lower 1/3's digested separately for several hours at 37°C in 0.2% collagenase/0.25% trypsin in HBSS. Following digestion, sterna were filtered through nitex mesh to remove residual tissue clumps and to facilitate the last stages of mechanical disaggregation. Cells were harvested by centrifugation, and resuspended in DMEM:F12/10% FBS medium at a final concentration of 2×10^5 cells/ml. Cultures were established by plating 4.5 mls of cell suspension per 60 mm Nunclon dish, or 0.75 ml per well of 12-well Nunclon plates. The plates were incubated overnight at 37°C to permit cell attachment, after which the feeding medium was supplemented with a final concentration of 14 mM Hepes, pH 7.2, 10 µg/ml ascorbic acid, and 1.0 -1.5% ethanol (v/v). The medium of parallel control cultures was supplemented with an equivalent volume of sterile distilled water. The plates were sealed with parafilm and incubated for 1 - 3 days at 37°C in a 5% CO₂ /95% air atmosphere without subsequent medium change, and then processed for histochemical staining (described below in 2.3.1), quantification of cartilage glycosaminoglycan formation (described below in 2.3.2), or analysis of cartilage-specific mRNA expression (described below in 2.6).

2.3 Histochemical Detection of Sulfated Glycosaminoglycans (GAGs)

2.3.1 Histochemical Staining with Alcian Blue Dye

To demonstrate the deposition of cartilage matrix proteoglycans, representative cultures of limb and facial prechondrogenic mesenchyme and sternal chondrocytes were collected at 1 - 3 days of incubation, medium was removed and cultures were stained overnight with 0.5% Alcian blue 8GX (Fluka or ICN Biochemicals) in 3% acetic acid, pH 1.0, according to the method of Hassell and Horigan (1982). Cultures were rinsed once in 3% acetic acid, pH 1.0, once in 3% acetic acid, pH 2.5, and once in distilled water to remove unbound dye. Some cultures were then air-dried and photographed. Alternatively, the Alcian blue dye was eluted and quantified (see below).

2.3.2 Quantification of Cartilage GAG Formation by Elution of Bound Alcian Blue Dye with Guanidinium Hydrochloride (GuHCl)

The extent of Alcian blue - positive cartilage matrix accumulation in some limb mesenchyme cell cultures and sternal chondrocyte cultures was quantified by measuring the amount of the sulfated GAG-bound dye according to the method of Hassell and Horigan (1982). Micromass limb mesenchyme cultures and sternal chondrocyte cultures were stained overnight with 0.5% Alcian blue 8GX, pH 1.0, and rinsed as described above in 2.3.1. To each culture well, 500 µl of 6M GuHCl was added. The

plates were then sealed with parafilm and incubated for at least 1 hour at room temperature on a rocking platform to elute the Alcian blue dye into the GuHCl. Duplicate 200 µl aliquots from each sample were transferred to individual wells of a 96-well microtiter plate. A dilution series of Alcian blue dye standards dissolved in 6M GuHCl was prepared in parallel. Absorbance of each sample was read at a wavelength of 600 nm on a Tecan Spectra II plate reader. A standard curve was obtained by plotting absorbance values of the standard series against their appropriate Alcian blue dye contents. The eluted dye content of each culture was calculated from the linear regression equation of the standard curve (InStat 2.01 software program) and normalized against the average DNA content of replicate parallel cultures. DNA quantification was performed as described below in 2.4.3.

2.4 Biochemical Analysis of Sulfated Glycosaminoglycan (GAG) Accumulation

2.4.1 Labeling of Cultured Cells with $^{35}\text{SO}_4$

To quantitatively analyze sulfated glycosaminoglycan accumulation in both the cell layer and culture medium, the medium of certain cultures was supplemented with 5 µCi/ml [^{35}S]H₂SO₄ (1000 Ci/mmol, carrier-free; Dupont NEN). Isotope was added at the time of initial feeding, and the cells were continuously labeled over a 3-day period of incubation. Following labeling, the medium from each well was collected, and the adherent cell layer scraped from the culture dish into 425 µl HBSS for subsequent analysis of ^{35}S incorporation into sulfated GAGs and determination of total cellular DNA content.

2.4.2 Measurement of GAG-incorporated $^{35}\text{SO}_4$ in Medium and Cell Layer Fractions of Limb and Facial Mesenchyme Cultures

While keeping each tube immersed in ice-cold water, cell layer samples were sonicated for 30 seconds, and a 200 µl aliquot removed for DNA analysis (see 2.4.3 below). The remaining 225 µl of each cell layer sonicate was then incubated in boiling water for 2 - 3 min., allowed to cool at room temperature, and 25 µl of a 0.02 g Pronase/5 ml 2M Tris pH 8.0 solution added to each boiled cell sonicate sample. A 200 µl aliquot of each medium sample was transferred to a separate tube, and 22 µl of the pronase solution added. Both cell layer and medium samples were incubated overnight at 55°C to ensure complete proteolysis. Following overnight incubation at 55°C, each of the above prepared samples were spotted onto individual 2.7 X 2.2 cm squares drawn

on a 11.6 X 10.7 cm sheet of Whatman 3 mm filter paper, and dried under a hot-air blower. After all samples were applied, the sheets of filter paper were soaked five times at 37°C for 30 minutes each in tanks containing 1% cetylpyridinium chloride (CPC)/0.3M NaCl. After the final soak, the filter paper sheets were dried in a vacuum oven for 2 hours at 80°C. Individual squares to which the cell layer- and medium fraction- sulfated GAG-CPC complexes remained bound were then cut from the sheets (Kosher, 1976; Kulyk et al., 1989b), and transferred to scintillation vials. Scintillation fluor (Ready Safe Fluor, Beckman) was added to each vial, and the GAG-bound ³⁵S label in each sample was determined by scintillation counting using a Beckman LS-9000 liquid scintillation counter. Radioactivity measurements for each sample were then normalized per µg of total DNA (as determined by DAPI-fluorometric analysis of the corresponding DNA aliquot, described below in 2.4.3).

2.4.3 Fluorometric DNA Quantification

Total cellular DNA content of limb and facial prechondrogenic mesenchyme cell cultures and sternal chondrocyte cultures was determined by a modification of the method of Brunk et al. (1979). 200 µl aliquots of limb and facial mesenchyme culture cell layer sonicates in HBSS, or 200 - 500 µl aliquots of sternal chondrocyte culture sonicates in 0.25% trypsin-EDTA were transferred to a glass tube and mixed with 2.5 - 3.0 ml DAPI solution (100 mM NaCl, 10 mM EDTA, 10 mM Tris, pH 7.0, and 100 ng/ml DAPI). A series of chicken DNA (Pharmacia) standards (0, 0.25, 0.5, 1.0, 2.0, and 4.0 µg total DNA in 2.5 - 3.0 ml DAPI solution) was prepared in triplicate. Samples and standards were equilibrated at room temperature for 1 hour, and then read on an AMINCO fluoro-microphotometer, or on an Optical Technologies Model A4 Fluorometer at 360 nm excitation wavelength and 450 nm emission wavelength. The standard curve was obtained by plotting fluorescence values of the chicken DNA standards against their corresponding µg DNA values. The µg DNA content of each cell sonicate sample was determined from the linear regression equation of the standard curve using InStat 2.01 software.

2.5 Analysis of Cellular Proliferation: Labeling of Cultured Cells by ³H-Thymidine

To quantitatively analyze cellular proliferation, the medium of some limb mesenchyme cultures was supplemented with 5 µCi/ml [methyl] ³H-thymidine (78.0 Ci/mmol; Amersham), and the cells continuously labeled for the remainder of a 3-day period of incubation. Following labeling, the medium from each well was discarded,

and the adherent cells detached by incubation in 200 μ l 0.25% trypsin-EDTA solution which was then transferred to scintillation vials. Scintillation fluor (Ready Safe Fluor, Beckman) was added to each vial, and the ^3H -thymidine label in each sample determined by scintillation counting using a Beckman LS-9000 liquid scintillation counter.

2.6 RNA Analysis

2.6.1 RNA Isolation

In initial experiments, cytoplasmic RNA was prepared from pooled limb mesenchyme cells of 5 - 20 high-density micromass cultures or 20 - 40 low-density mesenchyme spot cultures (i.e., 1 - 8 $\times 10^6$ cells) following a modification of the method of White et al. (1986), as previously described (Kulyk, 1991). Briefly, cells were suspended for 5 minutes on ice in 50 μ l of 10 mM Tris (pH 7), 1 mM EDTA containing 0.5% Nonidet P-40 and 10 mM vanadylribonucleoside complex. Nuclei were removed by centrifugation (2.5 minutes, 7000g), and the supernatant was mixed with 800 μ l of 7.6 M GuHCl, 0.1M potassium acetate (pH 5). Following sequential extractions with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), the aqueous phase was recovered by centrifugation. RNA was selectively precipitated by addition of 0.6 volumes of 95% ethanol, followed by overnight incubation at -20°C and centrifugation (30 minutes, 13,000g) (method of Cheley and Anderson, 1984).

In later experiments, total RNA was isolated from limb and facial prechondrogenic mesenchyme cell cultures, and from sternal chondrocyte cultures using TriZol™ Reagent (GIBCO/BRL). Spot cultures (typically 8 - 15 high density micromass cultures, or approximately 1 - 5 $\times 10^6$ cells) were rinsed twice with cold HBSS and, using a Teflon scraper, collected from the culture plate in 500 μ l TriZol reagent. The TriZol reagent containing the spot cultures was then mixed vigorously with 100 μ l chloroform, incubated at room temperature for 2 - 3 min., and centrifuged for 10 min at top speed in a clinical centrifuge. The upper aqueous layer was carefully transferred to a new 1.5 ml microtube, and an equal volume of isopropyl alcohol added. Each sample was vortexed and incubated for 10 min at room temperature, after which total RNA was recovered by a 10 min centrifugation at 12,000g, 4°C on a Heraeus Refrigerated Microcentrifuge. The RNA pellet was washed twice with cold 80% ethanol, briefly air-dried, dissolved in 20 - 50 μ l deionized formamide, and stored at -20 to -80°C for subsequent analysis.

2.6.2 Determination of Relative Amounts of Total Poly (A)⁺ RNA or 18S rRNA in Individual Samples

The relative amounts of total poly(A)⁺ RNA in individual samples were determined by hybridization with oligo(dT)₁₈ following the method of Harley (1987). Alternatively, the relative amounts of 18S rRNA/sample were determined by hybridization with an oligonucleotide complementary to chicken 18S rRNA (5'-TTCTCAGGCTCCCTCTCCGGAATCGAA-3') (Kulyk et al., 2000). The probes were 5' end-labeled for 45 min at 37°C with [γ^{32} P] ATP (3000 Ci/mmol, Amersham) using T4 polynucleotide kinase (10 units/ μ l; GIBCO 5' end-labeling kit), after which the unincorporated label was removed by passing the reaction mixture through a Sephadex G-25 spun column packed in a Costar spin-X 0.2 μ m micropore filtration unit. After denaturation for 15 min at 55°C in 6X SSC¹ and 7.5% formaldehyde, 1 - 2 μ l aliquots of the isolated RNA samples were spotted onto nitrocellulose (MSI Nitroplus) using a 96-well "BioDot"(Bio-Rad) vacuum filtration unit. The nitrocellulose blots were then baked in a vacuum oven for 1.5 - 2.0 hours at 80°C.

For detection of poly(A)⁺ RNA, blots were prehybridized at 25 - 30°C for at least 1 hour in a sealed plastic bag containing: 5X SSC, 5X Denhardt's solution², and 10 mM sodium phosphate, pH 6.5. The nitrocellulose blot was then hybridized overnight at 25 - 30°C in the same solution to which 50 pmol/ml labeled oligo (dT)₁₈ was added. Following hybridization, the blot was washed 4 times (10 min each) at 25 - 30°C in 2X SSC/0.1% SDS solution, wrapped in Saran Wrap cellophane, and exposed to Kodak X-OMAT AR autoradiography film at -80°C.

For detection of 18S rRNA, blots were prehybridized overnight at 42°C in a prehybridization solution of 50% formamide, 4X SSC, 50 mM sodium phosphate, pH 6.5, 1X Denhardt's solution, 0.1% SDS, and 100 μ g/ml denatured salmon sperm DNA. Hybridization was then carried out overnight at 55°C in a hybridization solution identical to the prehybridization solution, but containing 7.5 pmol of ³²P-labeled 18S rRNA-specific oligonucleotide, and 100 pmol/ml of cold 18S rRNA oligonucleotide. The blot was subsequently washed 4 times (5 min each) at 55°C in 2X SSC/0.1% SDS, then 4 times (15 min. each) at 55°C in 0.1X SSC/0.1% SDS, and exposed to Kodak X-OMAT AR film at -80°C. Relative ³²P-dT₁₈ or 18S rRNA hybridization signals were quantified by scanning the dots of the resultant autoradiographs at a wavelength of 540 nm using a Tecan Spectra II plate reader.

¹ 20 X SSC stock solution: 3 M NaCl, 0.34 M sodium citrate

² 50 X Denhardt's stock solution: 1% BSA, 1% Ficoll, 1% polyvinylpyrrolidone

2.6.3 Analysis of Gene-Specific mRNA Levels by RNA Dot-Blot and Northern Blot Hybridization

Aliquots of RNA samples containing equivalent amounts of total poly(A)⁺ RNA or 18S rRNA were spotted separately onto nitrocellulose using the 96-well BioDot apparatus. The nitrocellulose dot-blot was baked at 80°C in a vacuum oven for 90 min, then prehybridized overnight at 42°C. Blots that were to be later hybridized with BMPR-IB, TGF-β₂, TGF-β₄, Hsp 70, Hsp 47, and Hsp 90α cDNA probes were instead prehybridized overnight at 37°C. The pre-hybridization solution was comprised of 50% formamide, 4X SSC, 50 mM sodium phosphate, pH 6.5, 1X Denhardt's solution, 0.1% SDS, and 100 µg/ml denatured salmon sperm DNA. Hybridization was usually carried out overnight at 55°C in a solution identical to the prehybridization solution except for the addition of heat-denatured ³²P-labeled cDNA probes (described below). Blots to be probed with BMPR-IB, TGF-β₂ and TGF-β₄ were hybridized at 42°C, whereas the blots to be probed with Hsp cDNAs were hybridized at 50°C. The nitrocellulose dot-blot was washed 4 times (5 min each) at 42 - 55°C in 2X SSC/0.1% SDS, then 4 times (15 min each) at 42 - 55°C in 0.1X SSC/0.1% SDS, and exposed to Kodak X-OMAT AR film at -80°C. The levels of hybridizable RNA sequences were quantified by scanning the dots of the resultant autoradiographs at a wavelength of 540 nm using a Tecan Spectra II plate reader.

For Northern blots, RNA samples containing equivalent amounts of poly(A)⁺ RNA or total 18S rRNA (5 - 15 µg) were separated by electrophoresis through 1.2% agarose gels containing 6% paraformaldehyde in 1X MOPS buffer running at 5 V/cm of gel and blotted by upward capillary transfer onto supported nitrocellulose membranes. Membranes were baked at 80°C for 1.5 hours, hybridized to ³²P-labeled cDNA probes as described above, then washed under conditions of relatively high stringency, as described above.

2.6.4 Generation of cDNA Probes to Cartilage-Characteristic Genes

To determine the steady-state levels of various mRNAs, RNA dot-blot and Northern blots were hybridized with a panel of gene-specific ³²P-labeled cDNA probes. Probes for chicken type II collagen and aggrecan were cDNA plasmids pCAR2 (Vuorio et al., 1982) and ST-1 (Sai et al., 1986), respectively, provided by Drs. W. Upholt and M. Tanzer (University of Connecticut Health Center). My type X collagen probe was a cDNA plasmid, Splx (LuValle et al., 1988), provided by Dr. P. LuValle (University of Calgary). Plasmids containing cDNA inserts for TGF-β₂, TGF-β₃ and TGF-β₄ (Jakowlew et al., 1991) were obtained from Dr. S. Jakowlew (NIH). A cDNA plasmid

for Hsp70 (Morimoto et al., 1986) was provided by Dr. R. Morimoto (Northwestern University), and the cDNA probes for chicken Hsp47 (Pearson, Kulyk and Krone, unpublished) and Hsp90 α (Sass and Krone, 1997) were provided by Dr. P. Krone (University of Saskatchewan). Each of these probes was subsequently linearized by incubation at 37°C with an appropriate restriction endonuclease.

The probes for chicken *Sox-9*, BMPR-IB, *Erg*, *Ets-2*, and *Msx-2* were generated in our own laboratory by reverse transcriptase PCR (RT-PCR) using total RNA isolated from the limbs of stage 23 - 29 chick embryos (described below in 2.7). As reported in Kulyk et al. (2000), a partial cDNA for chicken *Sox-9* was generated by oligo (dT)₁₈-primed RT-PCR using the forward *Sox-9* primer, 5'-GTCCTCCTCAAGAGAACACCT-3', and the reverse *Sox-9* primer, 5'-TGTTCTTGCTGGATCCGTTTACC-3'. The resulting 168 bp PCR product was cloned into pCRII (Invitrogen) and sequenced at the DNA Sequencing Core Facility at the University of Calgary, which confirmed its identity with nucleotides 145 - 312 of the chicken *Sox-9* cDNA sequence reported by Healy et al. (1999) (GenBank accession #U12533). The *Sox-9* cDNA probe used in our hybridization analyses was generated from the *Sox-9* plasmid by PCR amplification using the *Sox-9* primers above and recognized a single transcript of approximately 3.5 kb on a Northern blot, in agreement with Healy et al. (1999).

Similarly, a partial cDNA for chicken BMPR-IB was generated using the forward primer, 5'-ACAGCTCCTTCTCGTGAAGAC-3', and the reverse primer, 5'-CGGAAAGCAACATGCATAAC-3'. The 251 bp PCR product was cloned into pCRII (Invitrogen) and sequenced, which confirmed its identity with nucleotides 1874 - 2125 of the chicken BMPR-IB cDNA sequence reported by Sumitomo et al. (1993) (GenBank accession #D13432). The BMPR-IB cDNA probe used in our hybridization analyses was generated from the BMPR-IB plasmid by PCR amplification using the BMPR-IB primers above.

A partial cDNA for chicken *Erg* was generated using the forward primer, 5'-GGCAACATCAAAGATGAGC-3' and the reverse primer, 5'-TCTTTGCGTTGCTTCTGGTA-3'. The 604 bp PCR product was cloned into pCRII (Invitrogen) and sequenced, which confirmed its identity with nucleotides 187 - 791 of the chicken *Erg* cDNA sequence reported by Dhordain et al. (1995). The *Erg* cDNA probe used in our hybridization analyses was generated from the *Erg* plasmid by PCR amplification using the *Erg* primers above and recognized a single transcript of approximately 3.5 kb on a Northern blot.

A partial cDNA for chicken *Ets-2* was generated using the forward primer, 5'-TCTGTGGGAACACCTGGAGCA-3', and the reverse primer, 5'-CGTGTCTCTAAG

TTTGCCAGA-3'. The 376 bp PCR product was cloned into pCRII (Invitrogen) and sequenced, which confirmed its identity with nucleotides 742 - 1118 of the chicken *Ets-2* cDNA sequence reported by Bouloukos et al. (1988) (GenBank accession #X07202). The *Ets-2* cDNA probe used in our hybridization analyses was generated from the *Ets-2* plasmid by PCR amplification using the *Ets-2* primers above and recognized a single transcript of approximately 4 kb, in agreement with Bouloukos et al. (1988).

A partial cDNA for chicken *Msx-2* was generated using the forward primer, 5'-CGCCTCACTGTACGGAACAT-3', and the reverse primer, 5'-CACTCTGAGGATCAGACTGT-3'. The 332 bp PCR product was cloned in pCRII (Invitrogen) and sequenced, which confirmed its identity with nucleotides 315 - 647 of the published chicken *Msx-2* cDNA sequence reported by Coelho et al. (1991). The *Msx-2* cDNA probe used in our hybridization analyses was generated from the *Msx-2* plasmid by PCR amplification using the *Msx-2* primers above, and recognized a single transcript of approximately 3 kb, in agreement with Coelho et al. (1991).

Approximately 25 - 50 ng of each of the above cDNA probes was labeled for 45 min. at 37°C with [α^{32} P] dCTP (3000 Ci/mmol, Amersham) by the random primed labeling method using Klenow DNA polymerase (Amersham or GIBCO/BRL Random Primer Labeling Kits). Unincorporated label was removed by passing the reaction mixture through a Sephadex G-25/G-50 spun column packed in a Costar spin-X 0.2 μ m micropore filtration unit.

2.7 Polymerase Chain Reaction (PCR)-Based Cloning Strategy

2.7.1 cDNA Synthesis

1 - 2 μ g of total RNA isolated from pooled wing buds of stage 23-29 chick embryos was reverse transcribed into cDNA using 200 units of Superscript RNase H⁻ reverse transcriptase (200 units/ μ l) in a 20 μ l reaction solution containing a final concentration of 1X 1st Strand Buffer (provided with enzyme, GIBCO), 0.01 M DTT, 0.5 mM dNTPs, and 100 pmol of oligo(dT)₁₈ primer for 1 hour at 42°C.

2.7.2 PCR-Amplification Reactions

The synthetic oligonucleotide primers described above in 2.6.4 were used in PCR-amplification reactions containing 2 μ l of the above cDNA reaction mixture as a template. Amplifications were carried out in a 100 μ l reaction solution containing 5 units Taq polymerase (5 units/ μ l; GIBCO/BRL), 1X Taq Buffer (provided with enzyme), 1.5 - 2.0 mM MgCl₂, 0.2 mM dNTPs, 20 pmol of each custom primer, and 10

µg BSA. The amplification conditions were 1 minute at 94°C; 30 cycles at 92°C, 30 seconds/ 60°C, 30 seconds/ 75°C, 90 seconds; 1 cycle of 5 minutes at 75°C in a MJ Research Thermocycler. The PCR products were then separated on 1.5% agarose gels in 1X TAE buffer (5 - 10 V/cm), stained with ethidium bromide (0.5 µg/ml) and examined by ultraviolet light.

2.7.3 T/A Cloning

PCR products were cloned into a modified EcoR1 site of the pCRII T/A vector (Invitrogen) according to manufacturer's instructions. Alternatively, circularized pCRII plasmid was prepared for T/A cloning by digesting 10 µg pCRII vector with the EcoRV restriction enzyme (GIBCO/BRL). The resulting digest was precipitated with 1/10 volume 2 M NaCl and 2.0 - 2.5 volumes 95% ethanol, and resuspended in 80 µl water. The resuspended vector was then incubated in a 100 µl reaction mixture containing a final concentration of 1X Taq Buffer, 1.5 mM MgCl₂, 2 mM dTTP, and 5 units Taq polymerase for 2 hours at 70°C to add "T" overhangs to the digested vector. Following incubation, the vector was purified by phenol/chloroform extraction, ethanol precipitated, and resuspended in 100 µl TE, pH 8.0.

The amount of PCR product needed to ligate with 50 ng of pCRII vector for a 1:1 molar ratio was calculated as follows:

$$\text{ng PCR product required} = \frac{\text{length of PCR product (bp)} \times 50 \text{ ng}}{3900 \text{ bp}^*}$$

*length of the pCRII vector

Ligation reaction mixtures contained the calculated amount of fresh PCR product, and a final concentration of 1X Ligation buffer (Invitrogen TA cloning kit; Ligation buffer, GIBCO), 50 ng pCRII vector, and 4 units T4 DNA Ligase (Invitrogen TA cloning kit; T4 DNA Ligase, GIBCO: 4 units/µl). Reactions were incubated overnight at 14°C.

For the transformation of plasmid DNA into bacterial hosts, INVαF' *E.coli* (Invitrogen TA cloning kit) were first grown overnight in 5 ml SOC medium (2.0% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂·6H₂O, 20 mM glucose) at 37°C in a rotary shaker water bath at 300 rpm. Growth phase INVαF' bacterial cultures were established by inoculating 5 ml fresh SOC medium with 100 µl of overnight INVαF' culture, and allowing this sub-culture to grow for 3 hours at 37°C at 300 rpm. Transformation-competent INVαF' bacteria were established by centrifuging the log-growth-phase bacterial suspension at 3000 rpm for 30 min.,

decanting the SOC medium, washing once with ice cold transformation buffer (TFB; 10 mM MES, 45 mM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 10 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 100 mM KCl, 3 mM hexamminecobalt chloride), and resuspending in 250 μl ice cold TFB. 35 $\mu\text{l}/\text{ml}$ of fresh DnD solution (1.53 g dithioreitol, 9 ml DMSO, 100 μl 1M potassium acetate, pH 7.5, H_2O to 10 mls) was then added to the cell suspension, and the sample placed on ice for 15 min. This last step was repeated once. Competent cells were dispensed into 50 μl aliquots which were combined with 2 μl 0.5 M β -mercaptoethanol (BME) and 2 μl of the ligation reaction mixture, and the sample placed on ice for 30 min. Competent cells and DNA were then heat-shocked for 2 min. at 42°C, and placed on ice for a further 2 min. Following heat-shock, 250 μl of room temperature SOC medium was added to the transformation mixture, and these were then transferred to a rotary shaker water bath (225 rpm) for 45 min. at 37°C. 50 - 100 μl of transformed INV α F' bacteria were then plated onto LB agar plates (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, 15g/L agar) containing 75 $\mu\text{g}/\text{ml}$ ampicillin, and 80 $\mu\text{g}/\text{ml}$ X-Gal (for blue/white color screening), and incubated overnight at 37°C.

Positive clones were selected and grown overnight in liquid medium containing 75 $\mu\text{g}/\text{ml}$ ampicillin for subsequent plasmid isolation (described below). Following plasmid isolation, clones were screened by PCR amplification using *Sox-9*-, *BMPR-IB*-, *Erg*-, *Ets-2*- and *Msx-2*-specific primers (listed above in 2.6.4), and the products subsequently separated on a 1.5% agarose gel. DNA was then purified from the remainder of selected amplification reaction mixtures using "Glassmilk" silica. To the DNA solution (up to 100 μl volume), 500 μl of 6 M NaI was added. Next, 10 μl (1 mg) of "Glassmilk" silica suspension (100 mg/ml Sigma silica in 3 M NaI) was added, and the DNA/NaI/silica mixture incubated for 5 min. at room temperature. Following incubation, samples were centrifuged for 20 sec. at 10,000 rpm, the supernatant discarded, and the pellet washed (100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA; mix solution with equal volume of 95% ethanol). This last step was repeated twice more before the pellet was dried and resuspended in 30 μl water. The suspension was incubated for 3 - 5 min. at 60°C to elute the DNA, then centrifuged for 1 min. at 13,000 rpm. An aliquot of the DNA-containing supernatant was run on a 1.5% agarose gel to determine the concentration, and the remainder of the sample utilized as ^{32}P -labeled probes in Northern RNA/dot blot analysis (described in 2.6.4).

Plasmids were isolated using a Qiagen MIDI prep kit according to manufacturer's instructions, or using the alkaline lysis method of mini prep plasmid isolation as described briefly here. Cultures of pCRII - containing INV α F' bacteria were grown in 5 ml SOC medium supplemented with 75 $\mu\text{g}/\text{ml}$ ampicillin. Bacteria were

allowed to grow overnight at 37°C in a rotary shaker water bath at 300 rpm. Bacteria in 1.5 ml of overnight culture were pelleted by centrifugation for 1 min. at 13,000 rpm, after which the SOC medium was removed, and the cells resuspended in 100 µl of resuspension solution (50 mM glucose, 25 mM Tris-HCl, pH 8.0, and 10 mM EDTA, pH, 8.0). Bacteria were lysed by the addition of 200 µl of ice cold denaturation solution (0.2 N NaOH, 1% SDS). Cytoplasmic components and chromosomal DNA were precipitated by the addition of 150 µl of neutralization solution (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, H₂O to 100 mls) and incubation on ice for 5 min. After centrifugation for 5 min. at 13,000 rpm, the supernatant was recovered and the plasmid DNA precipitated by addition of 2 - 2.5 volumes of absolute ethanol. Following incubation for 2 min. at room temperature, plasmid DNA was recovered by centrifugation (5 min. at 13,000 rpm), washed with 70% ethanol, and resuspended in a solution of 0.25 mg/ml RNase A. After 30 min. at room temperature, the plasmid solution was extracted with equal volumes of equilibrated phenol and chloroform, and the plasmid DNA precipitated by the addition of 1/10 volume 3M sodium acetate and 2 - 2.5 volumes of absolute ethanol. Following overnight incubation at -20°C, the purified DNA was recovered by centrifugation (13,000 rpm, 30 min.), and dissolved in 100 µl sterile dH₂O.

2.8 Differential Display Technique

The "Differential Display" PCR cloning method (Liang and Pardee, 1992) was utilized according to manufacturer's instructions (Display Systems Biotech) in an attempt to identify novel genes associated with ethanol-induced chondrogenesis. Following a modification of the Chomczynski and Sacchi procedure (1987), total RNA was isolated from low-density cultures of distal subridge limb mesenchyme that were incubated for 18 hours in the absence (control) or presence of 1.5% ethanol. Single-stranded cDNA was synthesized in fractions using 9 sets of poly A⁺ tail-anchored oligonucleotide primers ("downstream primers"; T₁₁VV, where V can be A, G, or C). The cDNA in each of the 9 preparations was then amplified using the same anchored primer previously used for reverse transcription, and a 10-mer oligonucleotide of arbitrary sequence. There were 24 of these "upstream" primers in total. Thus, 9 X 24 reactions were performed for each RNA sample (control versus ethanol). This was to ensure that the amplified sequences corresponded to the complete range of mRNA transcripts being expressed by the original mesenchyme cell populations. The resulting amplified cDNA fragments were then electrophoretically separated on 6% nondenaturing polyacrylamide gels, with candidate "differentially expressed" sequences

being identifiable as discrete bands. Fragments that behaved differently in control and ethanol-treated conditions were then eluted from the gel, reamplified, and cloned into a T/A vector. RNA dot-blot analysis, using the cloned fragments of interest as probes, was subsequently utilized to confirm that the isolated sequences were differentially expressed in control and ethanol-treated cultures of limb mesenchyme. If indeed differentially-expressed, the cDNA fragments would be sequenced, and a full length cDNA obtained.

2.9 Statistical Analysis

Statistical analyses, wherever applied in this thesis, were performed on a MacIntosh computer using the InStat Version 2.01 software program (student's unpaired *t*-test; two-sided ANOVA, Dunnet's Multiple Comparison test, Tukey's Multiple Comparison test, or Bonferroni's Multiple Comparison test).

3. RESULTS

3.1 *In Vitro* Effects of Aliphatic Alcohols on Cartilage Differentiation by Embryonic Limb Mesenchyme

3.1.1 Ethanol Effects on High-Density Micromass Cultures of Limb Mesenchyme

I first examined the ability of ethanol treatment to influence cartilage differentiation in high-density micromass cultures of embryonic chick limb mesenchyme cells. In an initial experiment, micromass cultures prepared from mesenchyme of whole stage 23/24 wing buds were incubated for 3 days in medium containing various concentrations of ethanol. The effects on cartilage matrix production were quantified by measuring $^{35}\text{SO}_4$ incorporation into sulfated glycosaminoglycans (GAG). As demonstrated in Figure 1A, concentrations of 0.2 - 2% ethanol (v/v) promoted a progressive dose-dependent increase in the accumulation of $^{35}\text{SO}_4$ -labeled GAG within limb mesenchyme cultures. At a medium concentration as low as 0.5% ethanol (approximately 85 mM), there was a statistically significant increase in pericellular sulfated GAG deposition relative to parallel cultures maintained in control medium ($P < 0.05$; ANOVA, Tukey's multiple comparison test). The stimulatory effect of ethanol on cartilage matrix formation was maximal at a concentration of 2.0% ethanol, which promoted a greater than four-fold increase in $^{35}\text{SO}_4$ -labeled GAG accumulation in the cell layer. Ethanol treatment also significantly elevated the amount of sulfated GAG secreted into the culture medium (Figure 1A), indicating that the presence of ethanol did not simply alter the relative proportions of soluble versus cell-layer-associated GAG produced by cultures. At chondrogenically stimulatory concentrations of 0.5 to 1.5%, ethanol had no significant effect on total DNA accumulation relative to untreated control cultures (Figure 1B). However, at ethanol concentrations exceeding 1.5%, there was a precipitous decline in the total DNA content of the micromass cultures (Figure 1B), reflecting the expected cytotoxic effects of alcohol at high doses. A treatment dose of 1.5 - 2.0% ethanol was selected for all subsequent experiments, since this yielded the maximal stimulatory effect on cartilage formation while only moderately reducing the final DNA content of micromass cultures.

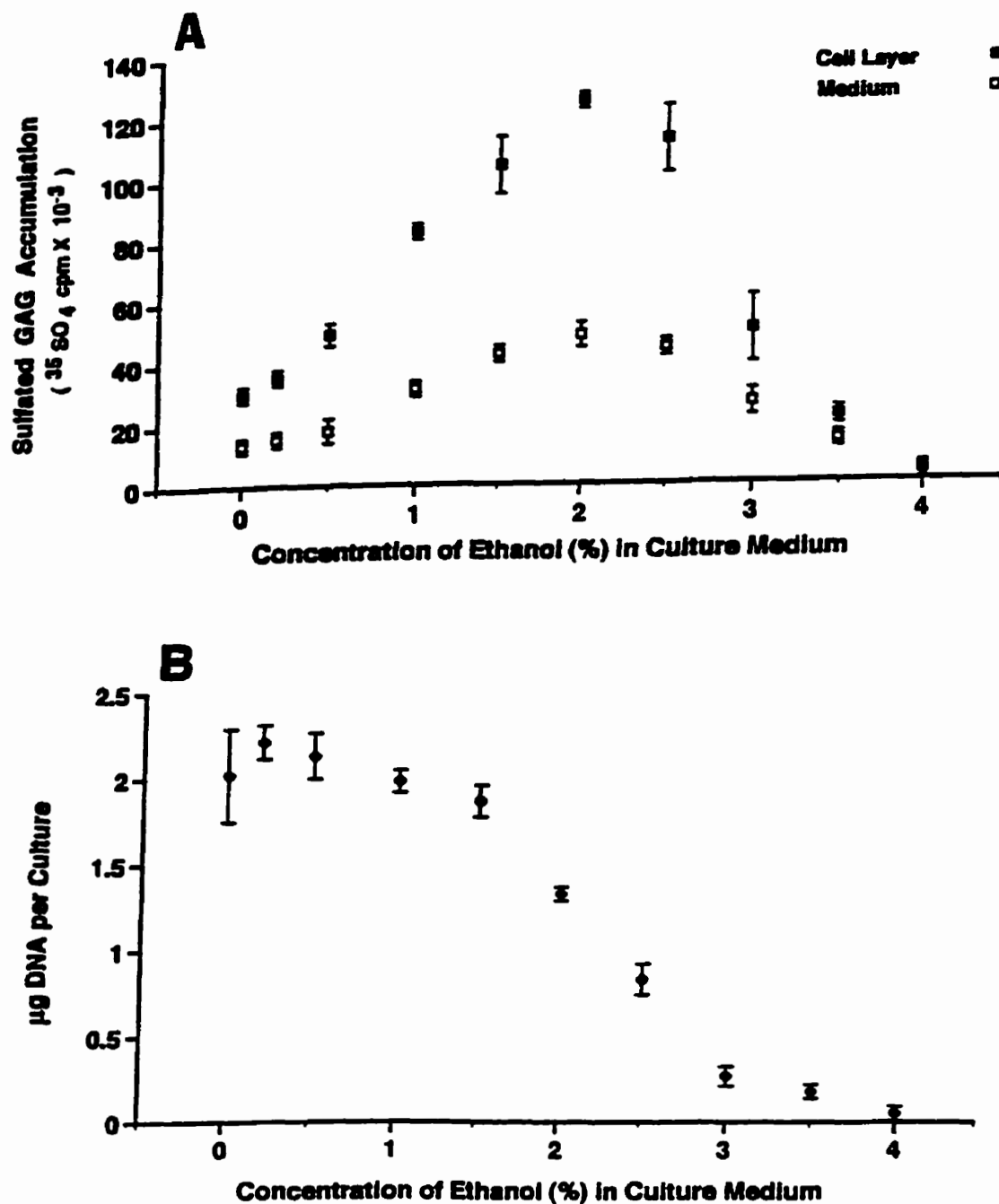


Figure 1 Effects of various concentrations of ethanol on sulfated glycosaminoglycan (GAG) accumulation and total DNA content in high-density micromass cultures of stage 23/24 chick embryo wing bud mesenchyme.

(A) To quantitatively analyze sulfated GAG accumulation, the culture medium was supplemented with $5 \mu\text{Ci/ml } ^{35}\text{SO}_4$ at the time of initial feeding, and the cells were continuously labeled over a 3-day period of incubation. Label incorporation into sulfated GAGs was measured in both the cell layer and culture medium. Data points represent the mean \pm standard deviation of determinations from four replicate cultures (eight replicates in the case of the 0% ethanol control group). (B) The total DNA content of the same cultures was determined by fluorometry. Data points represent the mean \pm standard deviation of at least four replicate cultures, as indicated above.

The stimulatory influence of ethanol treatment on cartilage differentiation was confirmed by histochemical staining with Alcian blue, a dye which binds to the sulfated proteoglycans of cartilage matrix (Figure 2). Following 2 days of incubation, matrix accumulation was only faintly discernible in untreated control cultures of stage 23/24 wing bud mesenchyme (Figure 2A), whereas cultures exposed to 2% ethanol revealed a precocious deposition of Alcian blue-positive matrix (Figure 2C). By 3 days of incubation, the control cultures had developed numerous discrete Alcian blue-positive cartilage nodules, separated by a considerable amount of nonchondrogenic tissue (Figure 2B). In contrast, the ethanol-treated cultures exhibited conspicuously greater matrix accumulation, forming a dense sheet of fused cartilage nodules that stained intensely with Alcian blue (Figure 2D).

It was possible that treatment effects observed in the foregoing experiments could have been influenced by the presence of myoblast progenitor cells in cultures prepared from whole wing bud mesenchyme. To circumvent this problem, we also examined the effects of ethanol exposure on micromass cultures prepared from mesenchyme dissected from the subridge region at the distal tip of the stage 24/25 chick embryo wing bud. This subridge mesenchyme contains a relatively homogeneous population of prechondrogenic cells that are uncontaminated by myoblast progenitors and have not yet initiated overt differentiative changes (Gay and Kosher, 1984). In control medium, the distal subridge mesenchyme undergoes progressive and uniform chondrogenic differentiation at micromass density, forming a virtually homogeneous sheet of Alcian blue-stainable cartilage tissue by 3 days of incubation (Gay and Kosher, 1984). When cultured in the presence of 2% ethanol, the subridge mesenchyme cells demonstrated an enhanced accumulation of Alcian blue-positive matrix during the initial 48 h of incubation (data not shown). By 3 days of incubation, both control and ethanol-treated cultures had formed continuous sheets of cartilage tissue that stained intensely with Alcian blue. However, quantitative biochemical analysis (Figure 3) confirmed that the total accumulation of $^{35}\text{SO}_4$ -labeled GAG per microgram of DNA in the cell layer and medium fractions of the ethanol-treated subridge mesenchyme cultures was elevated by approximately 1.5-fold relative to untreated control cultures (significant at $P < 0.05$). Since ethanol enhances cartilage matrix formation in homogeneous cultures of distal subridge mesenchyme, the alcohol appears to directly influence differentiation of the chondrogenic progenitor cells of the limb.

The stimulatory influence of ethanol on chondrogenic differentiation in limb mesenchyme micromass cultures was also evidenced by an accelerated accumulation of the mRNA transcripts for both type II collagen and aggrecan. Micromass cultures of

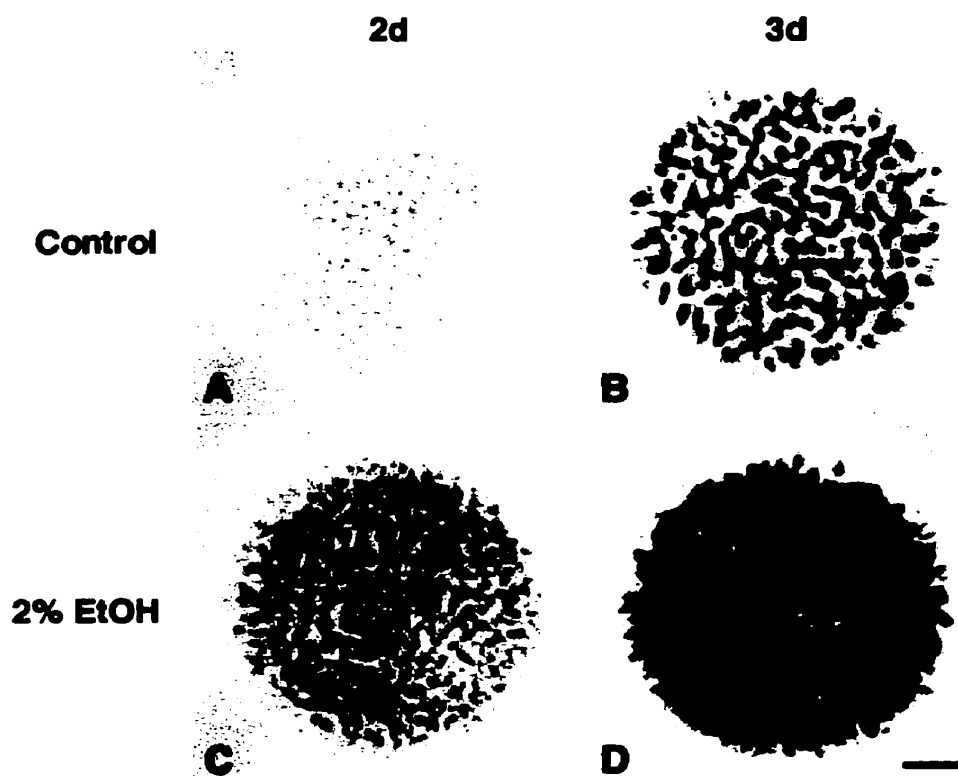


Figure 2 Alcian blue-positive matrix accumulation in ethanol-treated micromass cultures of stage 23/24 whole wing bud mesenchyme.

Cultures were stained with Alcian blue, pH 1, following 2 days (**A and C**) or 3 days (**B and D**) of incubation in either control medium (**A and B**) or medium supplemented with 2% ethanol (**C and D**). After 2 days of incubation, Alcian blue-positive cartilage matrix was barely detectable in control cultures (**A**), whereas the precocious appearance of stainable matrix was conspicuous in ethanol-treated cultures (**C**). By 3 days of incubation, control cultures formed numerous, discrete Alcian blue-positive nodules (**B**). Ethanol-treated cultures demonstrated a strikingly greater level of cartilage matrix accumulation (**D**), forming a sheet of fused nodules that stained intensely with Alcian blue. Scale bar, 1 mm for all four panels.

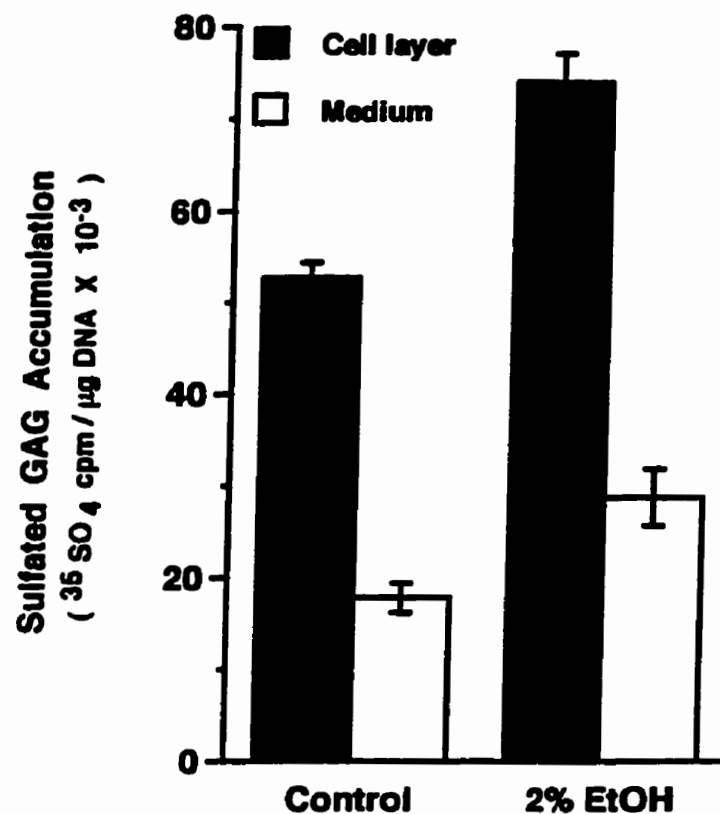


Figure 3 Sulfated GAG accumulation in ethanol-treated micromass cultures prepared from stage 24/25 wing bud distal subridge mesenchyme.

Levels of $^{35}\text{SO}_4$ -labeled glycosaminoglycan (GAG) accumulation were measured in both the cell layer and medium fractions of micromass cultures prepared from stage 24/25 distal subridge mesenchyme and incubated for 3 days in control medium or medium containing 2% ethanol. Error bars represent the mean \pm standard deviation of measurements from four replicate cultures.

stage 23/24 whole wing bud mesenchyme that were incubated with 2% ethanol demonstrated quantitatively higher levels of both type II collagen mRNA and aggrecan mRNA throughout a 3 day time course of *in vitro* chondrogenesis compared to parallel untreated control cultures (Figure 4). The stimulatory effect of ethanol on cartilage-characteristic gene transcript accumulation was most striking at 1 day of incubation, at which time control cultures expressed only faintly detectable levels of these cartilage-specific gene transcripts. In contrast, the alcohol-treated cultures expressed a 5-fold greater level of type II collagen mRNA and a 15-fold higher level of aggrecan mRNA at the same time point. Indeed, the aggrecan mRNA level was higher at 1 day of incubation in ethanol-treated cultures than at 3 days of culture in untreated cells (Figure 4). Ethanol exposure also induced precocious increases in type II collagen and aggrecan mRNA abundance in micromass cultures prepared from the distal subridge mesenchyme of stage 24/25 limbs (data not shown). Therefore, ethanol appears to act at a pretranslational level in stimulating the expression of the major cartilage matrix structural components. (All experiments described above were independently repeated at least once with qualitatively equivalent results, with the exception of the initial dose response experiment shown in Figure 1. Representative data are shown.)

3.1.2 Ethanol Effects on Low-Density Cultures of Limb Mesenchyme

The capacity of embryonic limb mesenchyme cells for spontaneous cartilage formation *in vitro* is dependent on a superconfluent plating density (Umansky, 1966; Ahrens et al., 1977). As previously reported (Kulyk et al., 1989b), when limb mesenchyme cells are plated at a reduced density of 0.5×10^5 cells per 10- μ l spot, the cells undergo relatively little spontaneous cartilage differentiation, and retain a predominantly mesenchymal or fibroblastic phenotype. We tested whether ethanol treatment could induce cartilage formation under these chondrogenically limiting, low-density culture conditions. Low-density cultures of stage 23/24 whole wing bud mesenchyme cells that were incubated for 3 days in control medium formed only sparse Alcian blue-positive foci that were restricted to the central area of the spot culture where the cell density was greatest (Figure 5A). In contrast, low-density cultures incubated in the presence of 2% ethanol developed numerous, intensely stained Alcian blue-positive chondrogenic foci that extended to the periphery of the spot cultures (Figure 5B). Quantitative analysis confirmed that ethanol treatment promoted a 14-fold increase in pericellular GAG deposition and a 9-fold increase in GAG

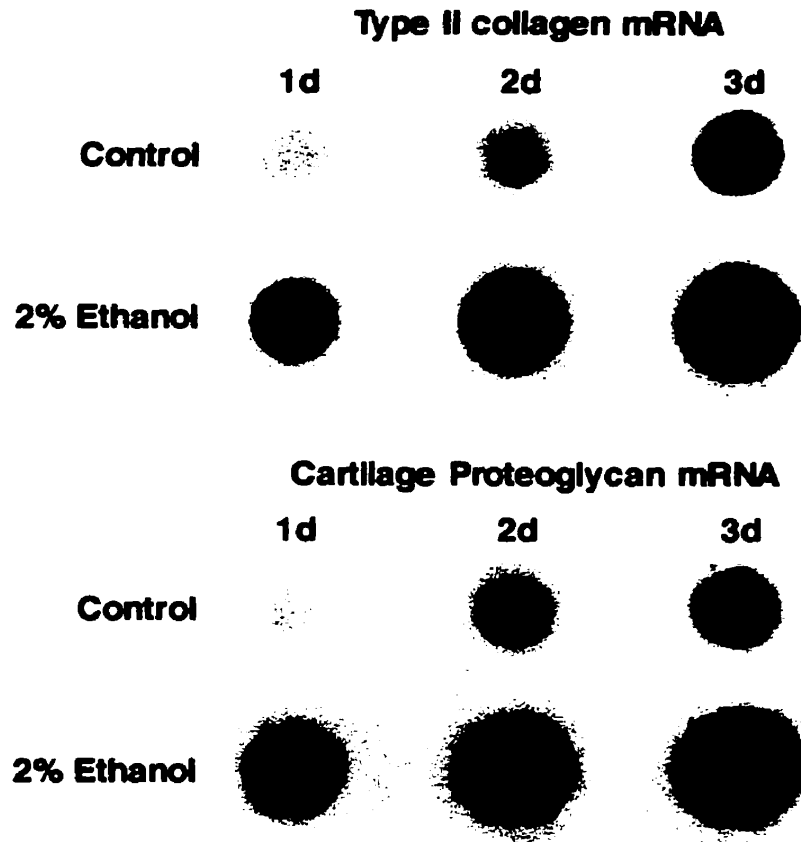


Figure 4 Dot-blot analysis examining the effects of ethanol exposure on type II collagen and aggrecan mRNA expression in micromass cultures of whole wing bud mesenchyme.

Total cytoplasmic RNA was isolated from high-density cultures of stage 23/24 whole wing bud mesenchyme cells that had been incubated for 1, 2, or 3 days in the absence (control) or presence of 2% ethanol. RNA dot-blot analysis was then hybridized with ^{32}P -labeled cDNA probes for type II collagen and aggrecan (cartilage proteoglycan), respectively. All RNA samples spotted on each of the two dot-blot analysis contained near equivalent amounts of total poly (A)⁺ RNA as determined by hybridization with ^{32}P -labeled oligo (dT)₁₈.

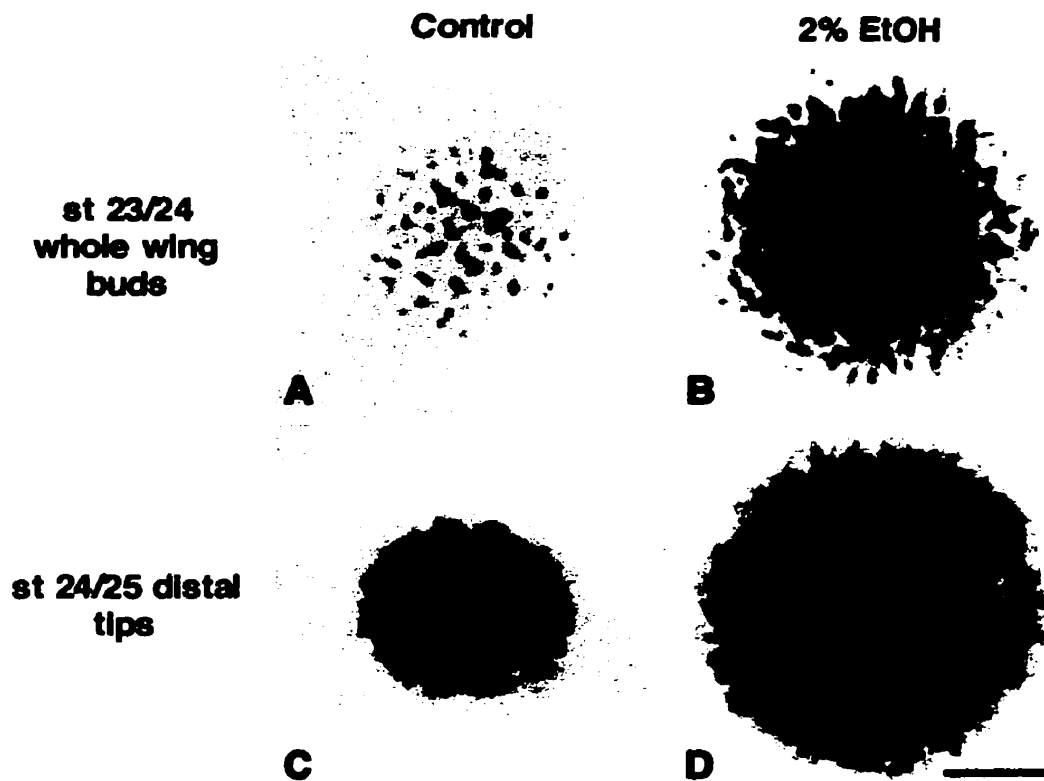


Figure 5 Alcian blue-positive matrix accumulation in ethanol-treated, low-density cultures of whole wing bud mesenchyme.

(A and B) Low-density cultures of stage 23/24 whole wing bud mesenchyme cells were stained with Alcian blue after 3 days of incubation in control medium (A) or in medium containing 2% ethanol (B). Control cultures formed only a few, faintly stained chondrogenic aggregates (A), whereas ethanol-treated cultures (B) developed numerous Alcian blue-positive foci of chondrogenically differentiating cells. **(C and D)** Low-density cultures of stage 24/25 distal subridge mesenchyme cells stained with Alcian blue after 3 days of incubation in the absence (C) or presence (D) of 2% ethanol. In control medium, cartilage matrix deposition was confined to the central area of the spot culture (C), whereas cells incubated in the presence of 2% ethanol (D) formed an extensive sheet of cartilage tissue extending to the periphery of the spot culture. (Low-density cultures were established by plating 0.5×10^5 mesenchymal cells in $10 \mu\text{l}$ medium). Scale bar, 1 mm.

secretion into the culture medium (Figure 6) (both significant at $P < 0.01$; unpaired t test). In addition, the ethanol treatment elevated cartilage-specific gene expression in low-density cultures of the stage 23/24 wing bud mesenchyme cells. Whereas type II collagen and aggrecan transcripts were barely detectable in control cultures after 3 days of incubation, ethanol treatment induced high levels of these cartilage-characteristic mRNAs (Figure 7A).

At the reduced plating density of 0.5×10^5 cells/10 μ l, stage 24/25 distal subridge mesenchyme cells displayed a modest level of spontaneous cartilage matrix formation when cultured for 3 days in control medium (Figure 5C). Ethanol exposure quantitatively elevated cartilage differentiation within low-density cultures of the distal subridge mesenchyme, as evidenced by enhanced Alcian blue-positive matrix deposition (Figure 5D) and by 2.3- and 1.9-fold increases in $^{35}\text{SO}_4$ -labeled GAG accumulation per microgram of DNA within the cell layer and culture medium, respectively (both significant at $P < 0.01$; data not shown). Levels of type II collagen and aggrecan mRNAs were elevated approximately 2-fold in ethanol-treated low-density cultures of distal subridge mesenchyme (Figure 7B). (All experiments described above were independently repeated at least once with equivalent results. Representative data are shown.)

3.1.3 Effects of Other Alcohols

Alcohols other than ethanol could also enhance cartilage differentiation in cultures of embryonic limb mesenchyme. Preliminary dose-response trials (data not shown) revealed that methanol, propanol and tertiary butanol (2-methyl-2-propanol) were all capable of enhancing Alcian blue-positive matrix accumulation in high-density micromass cultures of stage 23/24 whole wing bud mesenchyme cells, with optimal stimulation at doses of approximately 4, 1, and 1% (v/v), respectively (Figure 8). Quantitative biochemical analysis confirmed that these doses of the three alcohols promoted statistically significant increases ($P < 0.01$; ANOVA, Tukey's multiple comparisons test) in sulfated GAG accumulation in the cell layer and medium fractions of limb mesenchyme cells plated at either high density (Figure 9) or low-density (data not shown). Exposure to 1% tertiary butanol appeared to be as effective as 2% ethanol in stimulating *in vitro* chondrogenesis, elevating total GAG accumulation nearly 6-fold in high-density cultures (Figure 9) and nearly 12-fold in low-density limb mesenchyme cultures. Four percent methanol and 1% propanol appeared only slightly less potent than 2% ethanol in enhancing cartilage formation, eliciting 4- to 5-fold rises in total GAG accumulation in high-density micromass cultures (Figure 9) and 7- to 8- fold

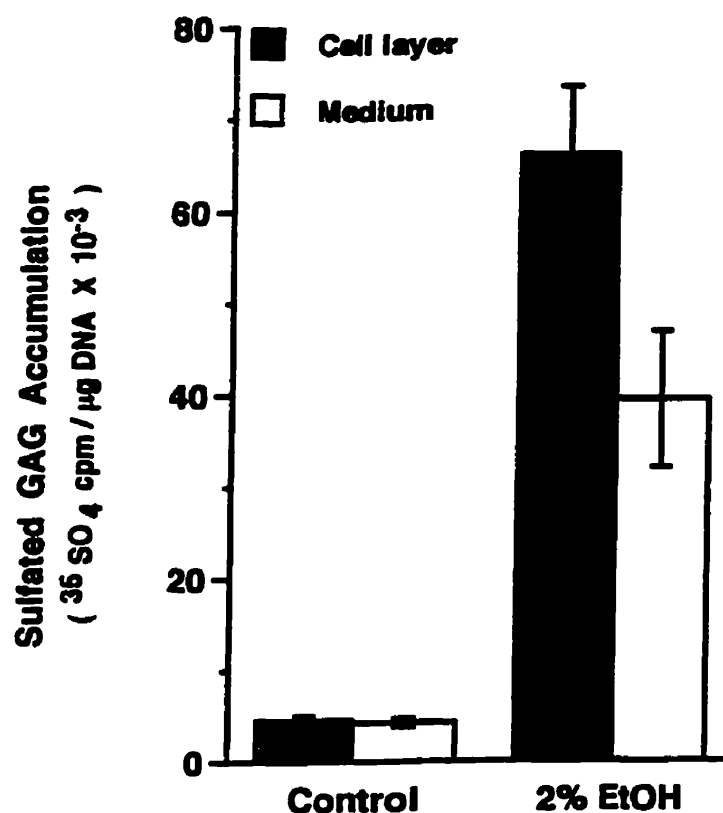


Figure 6 Sulfated GAG accumulation in ethanol-treated, low-density cultures of whole wing bud mesenchyme.

Levels of $^{35}\text{SO}_4$ -labeled GAG accumulation were measured in both the cell layer and culture medium fractions of low-density cultures prepared from stage 23/24 whole wing bud mesenchyme cells and incubated for 3 days in the absence (control) or presence of 2% ethanol. Error bars represent the mean \pm standard deviation of measurements from four replicate cultures.

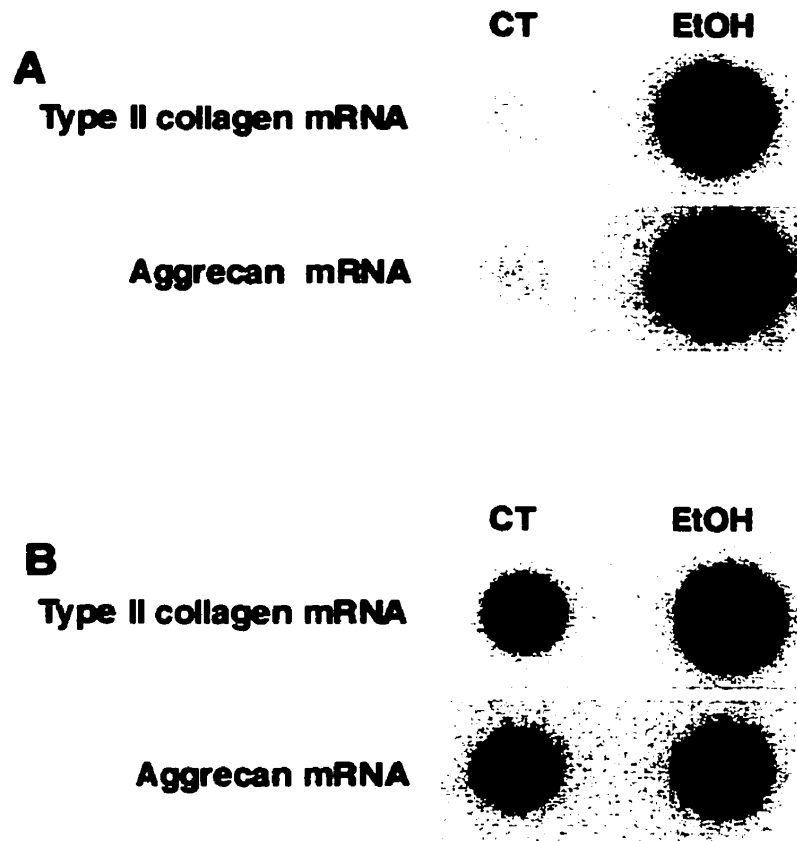


Figure 7 Dot-blot analysis examining the effects of ethanol treatment on type II collagen and aggrecan transcript expression in low-density cultures of limb mesenchyme.

Total cytoplasmic RNA was isolated from low-density cultures of stage 23/24 whole wing bud mesenchyme (A) and stage 24/25 distal subridge mesenchyme (B) following 3 days of incubation in control medium or in medium containing 2% ethanol. Within each horizontal row, the two samples spotted contain roughly equivalent amounts of total poly (A)⁺ RNA as determined by oligo (dT)₁₈ hybridization.

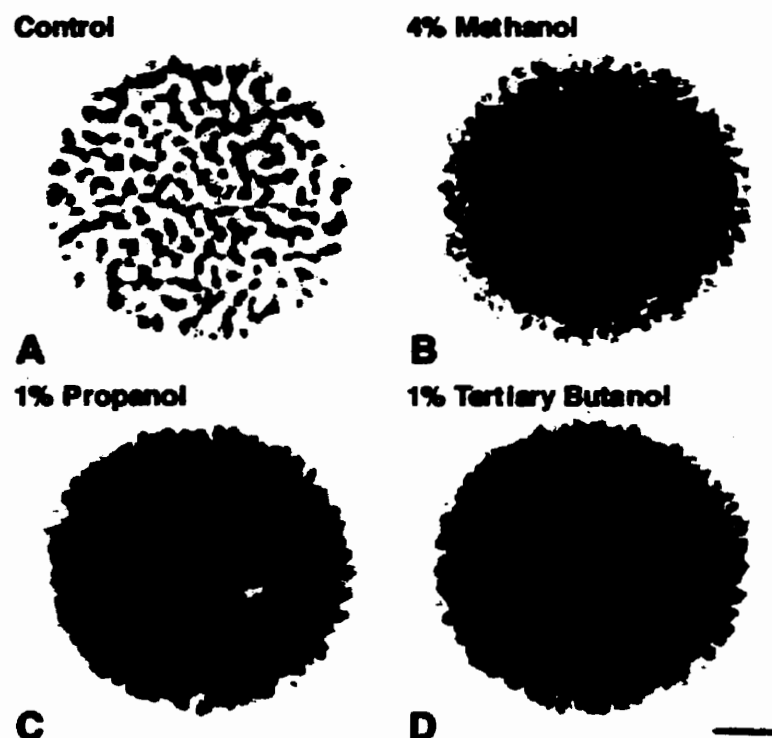


Figure 8 Alcian blue-positive matrix accumulation in limb mesenchyme cell cultures exposed to various aliphatic alcohols.

Micromass cultures of stage 23/24 wing bud mesenchyme were incubated for 3 days in control medium (A) or in medium supplemented with 4% methanol (B), 1% propanol (C), or 1% tertiary butanol (D). All three alcohol treatments (B, C, and D) markedly elevated Alcian blue-positive matrix formation relative to the level of spontaneous chondrogenesis in parallel control cultures (A). Scale bar, 1 mm for all four panels.

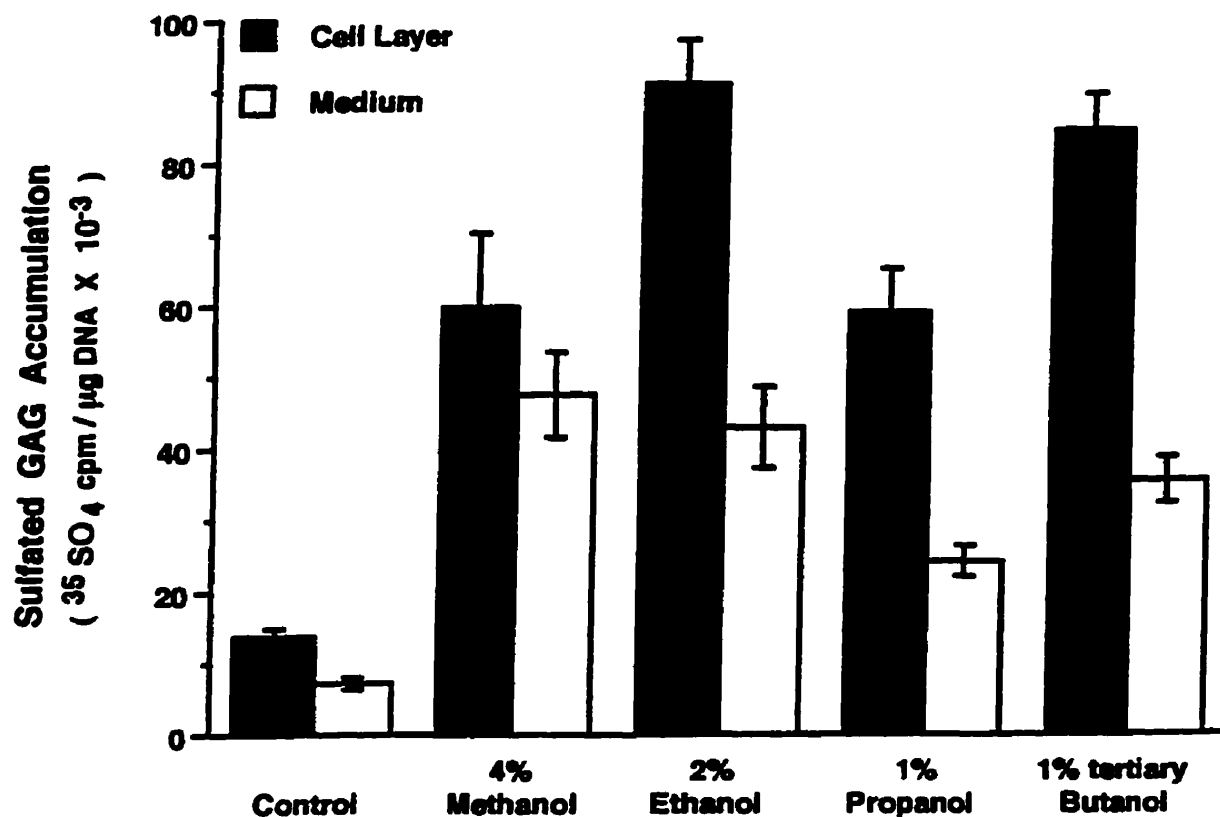


Figure 9 Influence of various aliphatic alcohols on sulfated GAG accumulation in micromass cultures prepared from whole wing bud mesenchyme.

Levels of $^{35}\text{SO}_4$ -labeled GAG accumulation were measured in the cell layer and culture medium fractions of high-density cultures of stage 23/24 wing bud mesenchyme cells that were incubated for 3 days in control medium or in medium containing 4% methanol, 2% ethanol, 1% propanol, or 1% tertiary butanol. Error bars represent the mean \pm standard deviation of measurements from four replicate cultures.

increases in low-density cultures. Dot-blot hybridization analysis confirmed that tertiary butanol treatment enhanced the expression of both type II collagen and aggrecan RNA transcripts in cultured limb mesenchyme cells (data not shown) in a manner similar to ethanol. The effects of methanol and propanol treatments on cartilage-characteristic mRNA levels were not examined. (Each of the above experiments was also independently repeated at least once with equivalent results. Representative data are shown.)

3.1.4 Effects of Ethanol on Proliferation of High- and Low-Density Cultures of Prechondrogenic Limb Mesenchyme

In many cell types there is an inverse relationship between cell proliferation and cell differentiation - i.e., as cells begin to differentiate, proliferation declines or ceases completely. As such, we questioned whether ethanol's ability to induce chondrogenic differentiation in cultured limb mesenchyme might be secondary to a suppressive effect on cellular proliferation. Alternatively, ethanol might stimulate cell proliferation and indirectly enhance chondrogenesis by increasing effective cell density. Since chondrocyte differentiation *in vitro* is density-dependent, an increase in the total number of cells might secondarily promote chondrogenesis. Therefore, experiments were performed to determine whether ethanol treatment affects the proliferation of prechondrogenic limb mesenchyme cells in culture, and whether ethanol's stimulatory effect on *in vitro* chondrogenesis is due to an effect on cell proliferation.

High-density micromass cultures of whole limb bud mesenchyme were incubated in the presence or absence of 1.5% ethanol, and cellular proliferation was monitored by ^3H thymidine uptake (an indicator of DNA synthesis). Some cultures were co-treated with 0.5 $\mu\text{g}/\text{ml}$ cytosine arabinoside, an agent previously shown to inhibit DNA synthesis in micromass culture (Biddulph et al., 1991). As demonstrated in Figure 10, treatment with 1.5 % ethanol alone had no significant effect on the level of ^3H -thymidine incorporation in the cultured limb mesenchyme relative to untreated control cultures ($P > 0.05$; ANOVA, Tukey's multiple comparisons test). As anticipated, the presence of cytosine arabinoside significantly reduced ^3H -thymidine uptake in both the presence and absence of ethanol. The cytosine arabinoside-treated cultures revealed a pronounced reduction in total Alcian blue-stainable matrix accumulation relative to control cultures (Figure 11A, C), consistent with the expectation that cell proliferation and therefore total cell numbers would be markedly reduced. (Indeed, when Alcian blue-positive matrix accumulation is normalized against total cellular DNA, the values for cytosine arabinoside-treated cultures are moderately

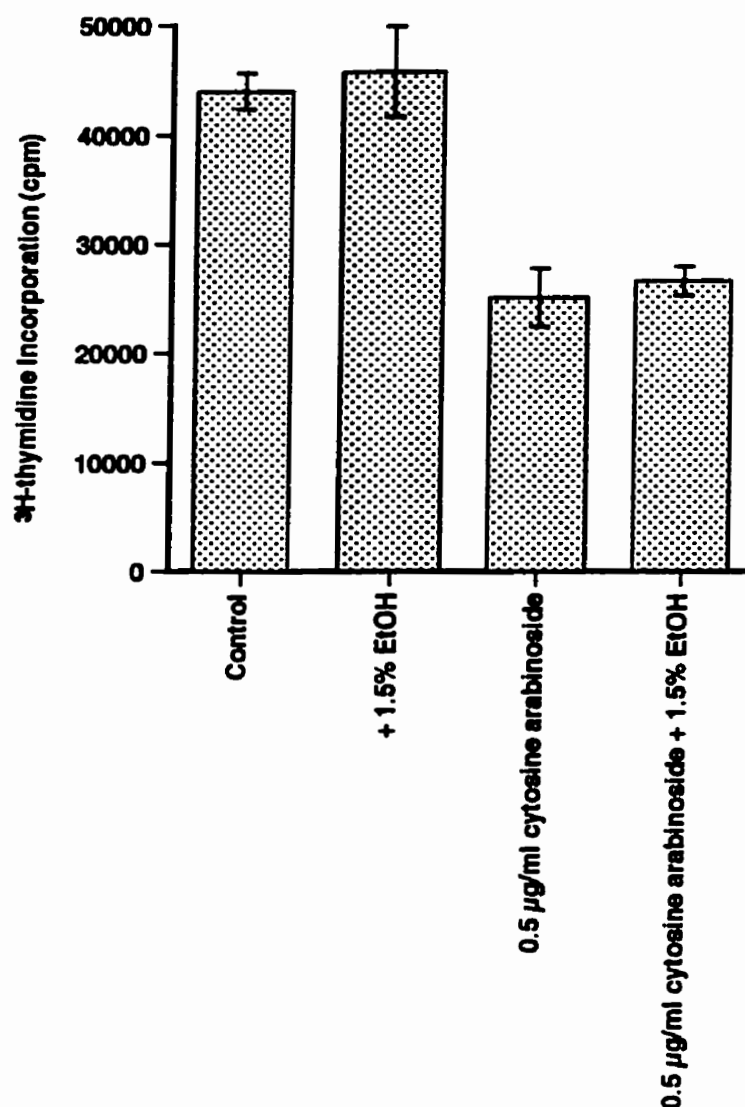


Figure 10 Effects of ethanol and the DNA synthesis inhibitor, cytosine arabinoside, on cellular proliferation in micromass cultures of stage 23/24 whole wing bud mesenchyme.

To quantitatively analyze cell proliferation, the culture medium was supplemented with ³H-thymidine, and the cells labeled continuously during a 3-day period of culture. Cultures were incubated in control medium, medium containing either 1.5% ethanol or 0.5 µg/ml cytosine arabinoside, or medium containing both ethanol and cytosine arabinoside. Bars represent the mean \pm standard deviation of measurements from four replicate cultures.

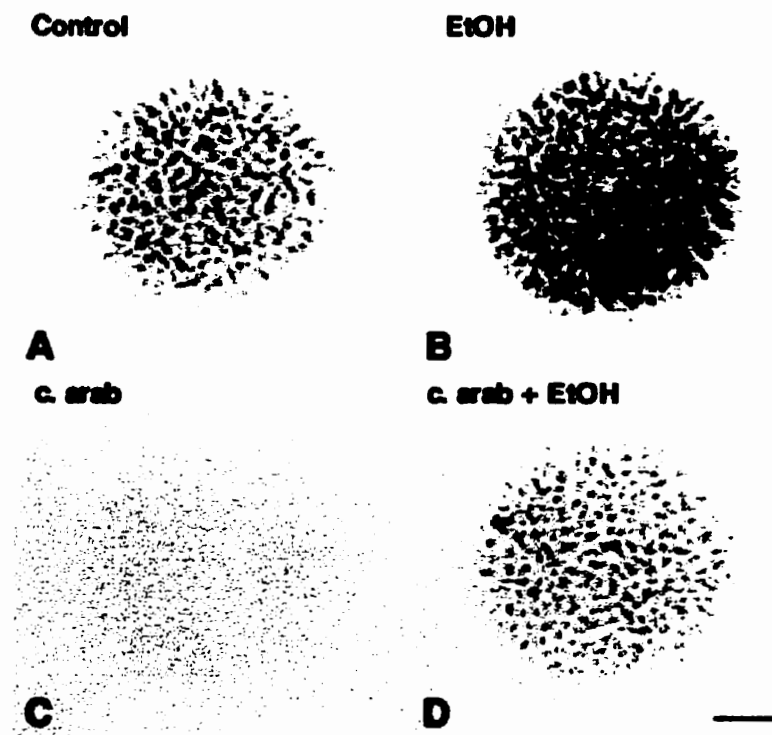


Figure 11 Photomicrographs demonstrating effects of cytosine arabinoside on ability of ethanol to enhance Alcian blue-positive matrix accumulation in micromass cultures of stage 23/24 whole wing bud mesenchyme.

Cultures were stained with Alcian blue, pH 1, following 3 days of incubation in control medium (A), medium supplemented with either 1.5% ethanol (B) or 0.5 μ g/ml cytosine arabinoside (C), or medium supplemented with both ethanol and cytosine arabinoside (D). Cytosine arabinoside treatment did not block the ability of 1.5% ethanol to stimulate cartilage differentiation in micromass cultures of prechondrogenic limb mesenchyme. Scale bar, 1 mm.

higher than those of controls; see Figure 12. The reason that ^3H -thymidine uptake was only partially blocked by cytosine arabinoside treatment may have been that the ^3H -thymidine label was introduced at the same time as the cytosine arabinoside rather than following a pre-incubation with cytosine arabinoside alone). Importantly, the suppression of cellular proliferation by cytosine arabinoside treatment did not block the ability of 1.5% ethanol to stimulate cartilage matrix synthesis in limb mesenchyme micromass cultures (Figures 11D and 12). Similar results were obtained in three independent experiments. These results suggest that, at a dose which effectively stimulates *in vitro* chondrocyte differentiation, ethanol treatment does not significantly alter the level of cell proliferation in micromass culture. Moreover, active cell proliferation may not be required for ethanol to exert its stimulatory effects on chondrogenesis.

These experiments were also carried out in low-density cultures of whole limb bud mesenchyme, which as previously reported, undergo relatively little cartilage differentiation spontaneously. As in high-density micromass cultures, ethanol did not alter the incorporation of ^3H -thymidine-label into limb mesenchyme cells plated at subconfluent density (Figure 13) ($P > 0.05$). In addition, histochemical staining with Alcian blue dye (Figure 14), and quantitative analysis of Alcian blue-positive cartilage matrix accumulation/ μg DNA (Figure 15) demonstrate that even under these chondrogenically limiting, low-density culture conditions, ethanol is still capable of exerting its stimulatory actions on cartilage differentiation in the presence of cytosine arabinoside (Figure 14D and Figure 15) (significant at $P < 0.001$).

3.1.5 Effect of Medium Serum Concentrations on Ethanol-Responsiveness of Limb Mesenchyme Cell Cultures

The above data demonstrate that chronic exposure to ethanol, or other short-chain aliphatic alcohols, enhances cartilage differentiation in cultures of embryonic chick limb mesenchyme cells. The tissue culture medium in these experiments contained a high concentration (10%) of fetal bovine serum (FBS) which is believed to contain a variety of polypeptide growth factors, hormones, and other soluble molecules (Freshney, 1987; HyClone Laboratories, personal communication) that have been implicated as potential regulators of chondrogenic differentiation (reviewed in Cancedda et al., 1995). Indeed, in most cases, cartilage formation is difficult to induce or maintain when defined or serum-free tissue culture medium is used (Kujawa et al., 1989; Bohme et al., 1992; Quarto et al., 1992; Paulsen et al., 1988). Therefore, we questioned whether ethanol's stimulatory effects on cartilage differentiation *in vitro* were dependent on the presence of high concentrations of serum. As shown in Table 1,

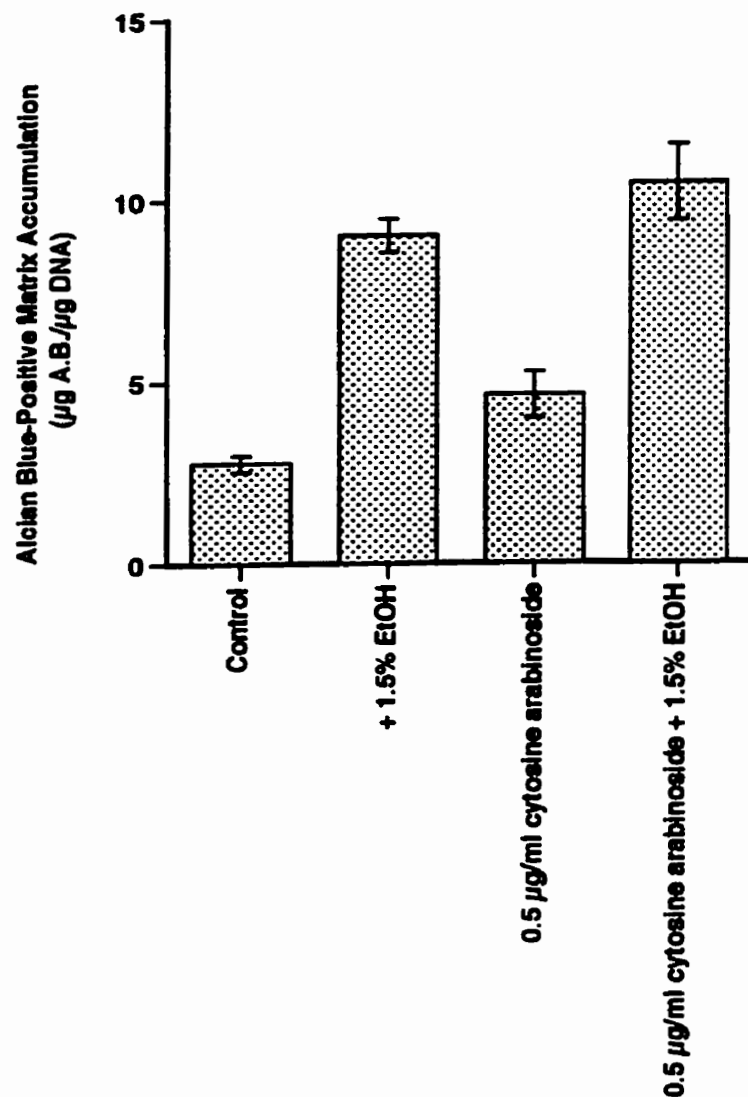


Figure 12 Sulfated GAG accumulation in micromass cultures of stage 23/24 whole wing bud mesenchyme cells that have been exposed to ethanol and/or cytosine arabinoside.

Cartilage GAG formation was quantified by elution of bound Alcian blue dye with GuHCl. The eluted dye content of each sample was then normalized against the average DNA content of parallel cultures. Cultures were prepared from stage 23/24 whole wing bud mesenchyme and incubated for 3 days in control medium, medium containing either 1.5% ethanol or 0.5 µg/ml cytosine arabinoside, or medium containing both ethanol and cytosine arabinoside. Bars represent the mean \pm standard deviation of measurements from four replicate cultures.

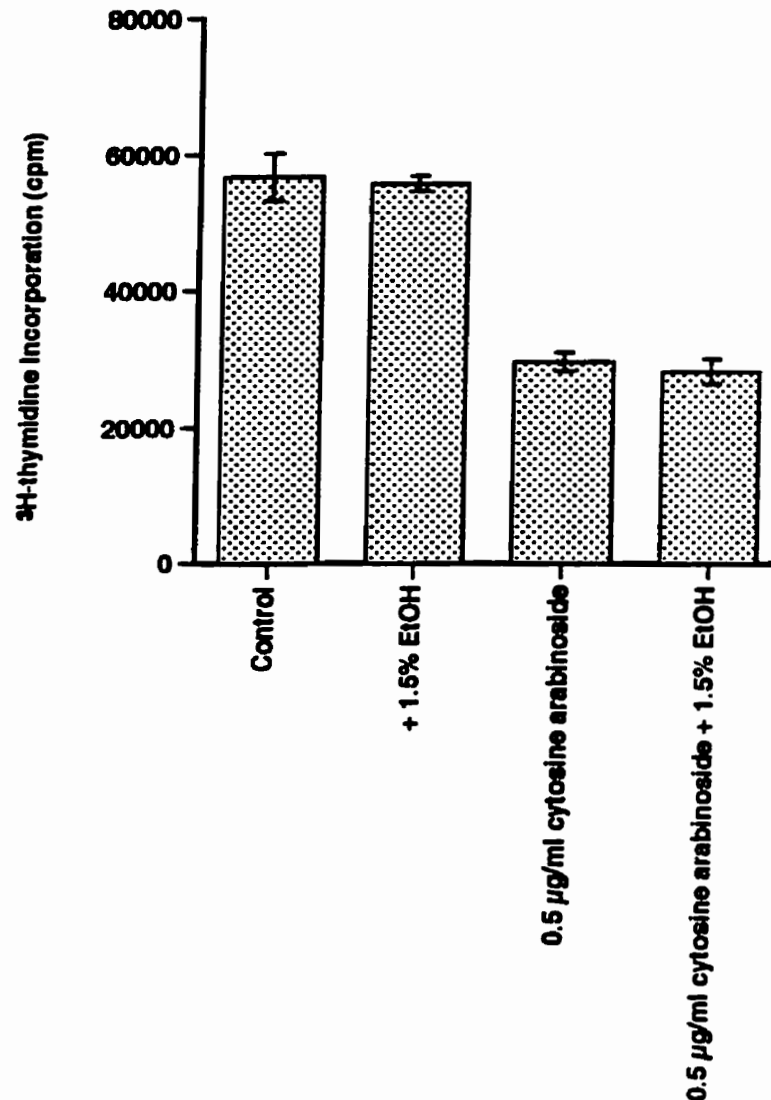


Figure 13 Effects of ethanol and cytosine arabinoside on ³H-thymidine uptake in low-density cultures of limb mesenchyme.

Levels of ³H-thymidine incorporation were measured in cultures of stage 23/24 whole wing bud mesenchyme cells plated at subconfluent density, and incubated for 3 days in control medium, either ethanol- or cytosine arabinoside-treated medium, or medium containing both of these agents. Treatment with 1.5% ethanol did not significantly alter cell proliferation in low-density cultures of limb mesenchyme. Bars represent the mean \pm standard deviation of measurements from four replicate cultures.

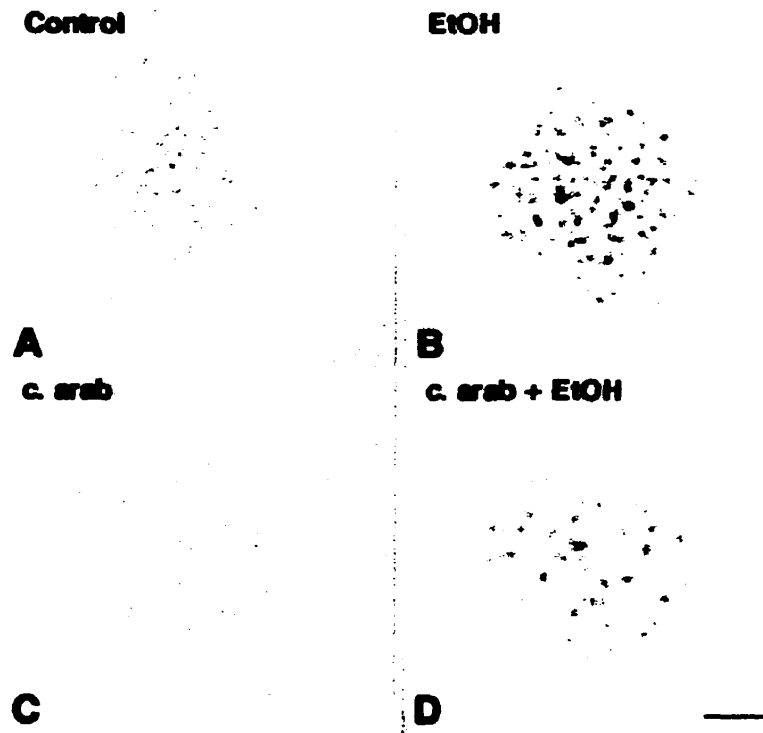


Figure 14 Photomicrographs demonstrating effects of cytosine arabinoside on ability of ethanol to enhance Alcian blue-positive cartilage matrix accumulation in low-density cultures of whole wing bud mesenchyme.

Cultures were stained with Alcian blue, pH 1, following 3 days of incubation in control medium (A), medium supplemented with either 1.5% ethanol (B) or 0.5 μ g/ml cytosine arabinoside (C), or medium supplemented with both ethanol and cytosine arabinoside (D). Cytosine arabinoside treatment did not block the ability of 1.5% ethanol to stimulate cartilage differentiation in chondrogenically-limiting, low-density cultures of limb mesenchyme. Scale bar, 1mm.

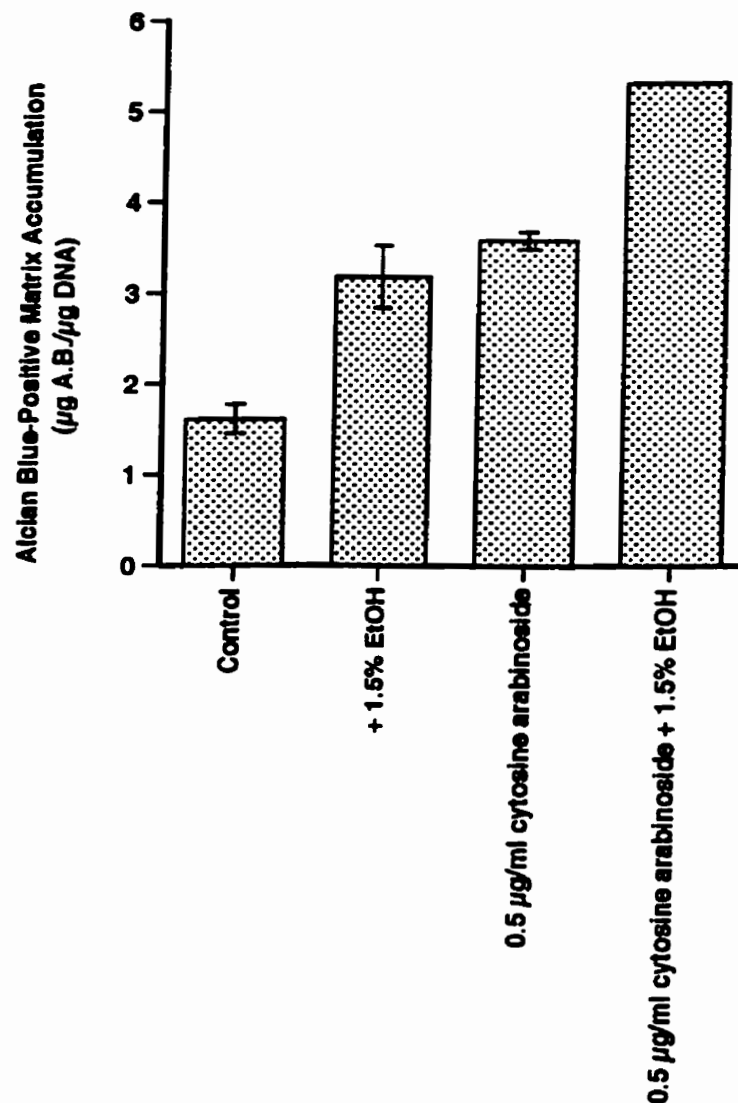


Figure 15 Quantitation of sulfated GAG accumulation in low-density cultures of limb mesenchyme cells that have been exposed to ethanol and/or cytosine arabinoside.

Levels of Alcian blue-positive matrix accumulation/ μg DNA were analyzed in subconfluent cultures prepared from stage 23/24 whole wing bud mesenchyme. Cultures were incubated for 3 days in control medium, medium containing either 1.5% ethanol or 0.5 $\mu\text{g}/\text{ml}$ cytosine arabinoside, or medium containing both ethanol and cytosine arabinoside. Bars represent the mean \pm standard deviation of measurements from four replicate cultures.

Table 1 Effect of medium serum concentration on ethanol-responsiveness of high-density cultures of mesenchyme cells isolated from whole stage 23/24 wing buds.

	<u>Total μg A.B. dye</u>	<u>Total μg DNA</u>	<u>μg A.B./μg DNA</u>
<u>1% Serum</u>			
Control	1.75 +/- 0.20	0.90 +/- 0.18	1.95 +/- 0.23
1.5% Ethanol	2.0 +/- 0.00	0.63 +/- 0.14	3.17 +/- 0.00
<u>10% Serum</u>			
Control	8.56 +/- 0.63	1.71 +/- 0.03	5.01 +/- 0.37
1.5% Ethanol	20.88 +/- 4.54	1.02 +/- 0.17	20.50 +/- 4.00

Values given indicate mean +/- standard deviation from four replicate cultures.

ethanol treatment promoted a 2.4-fold increase in total Alcian blue-positive matrix accumulation in high-density micromass cultures of whole wing bud mesenchyme incubated in the presence of 10% serum. In comparison, the ability of ethanol to enhance chondrogenesis was greatly diminished at a reduced serum concentration of 1%. This result suggests that certain components of FBS are indeed required for ethanol to exert its stimulatory effects on *in vitro* chondrogenesis.

3.2 Alcohol Effects on *In Vitro* Cartilage Differentiation by Embryonic Facial Mesenchyme

Whereas the prechondrogenic mesenchyme cells of the embryonic limb derive from somatic mesoderm, the vertebrate facial skeleton (including the frontal bone, nasal septum, maxilla, hard palate, mandible, and hyoid apparatus) differentiates entirely from neural crest ectoderm. Moreover, a high incidence of facial dysmorphologies is associated with gestational ethanol exposure in both humans and laboratory animals (Webster and Ritchie, 1991; Weston et al., 1994). As such, experiments were performed to determine whether ethanol treatment could induce stimulatory effects on cartilage differentiation by ectodermally-derived prechondrogenic mesenchyme cells of the facial primordia as it does in mesoderm-derived prechondrogenic mesenchyme cells of the limb.

3.2.1 Effects of Ethanol Treatment on Micromass Cultures of Facial Mesenchyme

Micromass cultures of facial mesenchyme cells isolated from the frontonasal, maxillary, mandibular and hyoid processes of the stage 24/25 chick embryo display qualitatively distinct patterns of spontaneous chondrogenic differentiation during a 3 day period of incubation (Wedden et al., 1986; Kulyk and Reichert, 1992). Mesenchyme cells isolated from the frontonasal and mandibular processes exhibit relatively high levels of spontaneous cartilage differentiation when cultured in control medium. Specifically, the frontonasal process mesenchyme forms irregularly shaped patches or sheets of differentiating chondrocytes that elaborate an Alcian blue-stainable matrix containing sulfated glycosaminoglycans (GAG) (see Figure 16A). Mandibular process mesenchyme develops numerous spheroidal, Alcian blue-positive nodules of chondrogenically differentiating cells which are separated by nonchondrogenic tissue (Figure 16C). In contrast, mesenchymal cells from the maxillary processes and hyoid arches elaborate little or no Alcian blue-positive cartilage matrix during a 3 day period of culture in control medium (Figures 16B,D).

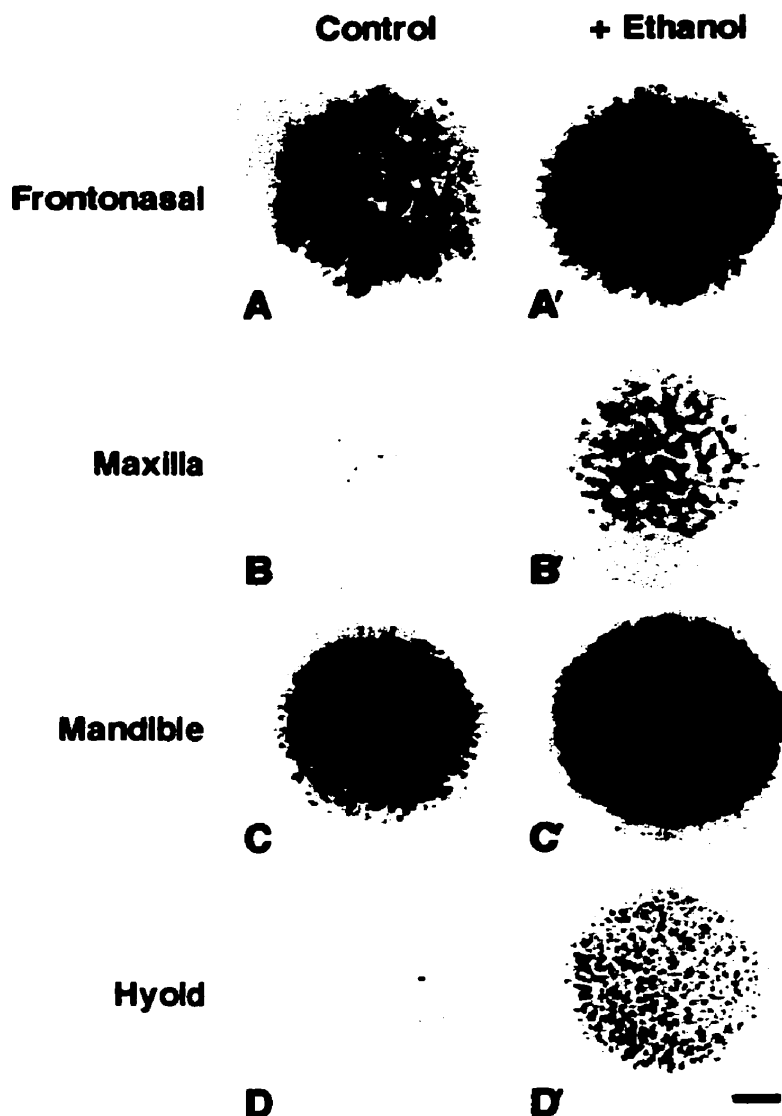


Figure 16 Alcian blue-positive matrix accumulation in ethanol-treated micromass cultures of stage 24 chick embryo facial mesenchyme.

Cultures of both frontonasal process (A) and mandibular process (C) mesenchymes spontaneously developed numerous Alcian blue-positive chondrogenic cell aggregates following a 3-day incubation in control medium. Continuous exposure to 1.0% ethanol markedly elevated cartilage matrix production in the frontonasal (A') and mandibular (C') mesenchyme cultures, yielding sheets of cartilage tissue that stained intensely with Alcian blue. Control cultures of maxillary (B) and hyoid process (D) mesenchymes formed little or no Alcian blue-stainable cartilage matrix. In contrast, the presence of 1.5% ethanol induced the differentiation of numerous Alcian blue-positive chondrogenic cell aggregates in the maxillary and hyoid mesenchyme cultures (B', D')

Scale bar, 1 mm for all photographs.

Initial experiments examined the effects of various ethanol concentrations on the incorporation of $^{35}\text{SO}_4$ -label into sulfated GAG, and on total DNA accumulation in micromass cultures prepared from mesenchyme cells of the frontonasal, maxillary, mandibular and hyoid primordia. In all four facial mesenchyme populations, the presence of ethanol in the culture medium promoted a dose-dependent increase in total sulfated GAG accumulation relative to parallel cultures incubated in control medium (Figure 17). In frontonasal, maxillary, and mandibular mesenchyme cultures, statistically significant elevations of sulfated GAG accumulation were observed with as little as 0.5% (v/v) ethanol ($P < 0.01$; ANOVA, Dunnet's multiple comparisons test). Maximum stimulation of GAG production was observed at doses of 1% ethanol in cultures of frontonasal and mandibular mesenchyme (8.6- and 2.0-fold higher than control cultures, respectively) and at 1.5% ethanol in maxillary mesenchyme (3.4-fold increase). In cultures of hyoid mesenchyme, concentrations of 1.5 - 2.0% ethanol promoted over 4-fold increases in sulfated GAG production (significant at $P < 0.05$).

Ethanol had variable effects on DNA accumulation in the facial mesenchyme cultures. There was a notable reduction in total DNA accumulation relative to control cultures in frontonasal and mandibular mesenchyme treated with 1.5 - 2.0% ethanol, and in maxillary and hyoid mesenchyme exposed to 2.0% ethanol (Figure 17). Lower concentrations of ethanol moderately increased the DNA content of frontonasal and maxillary cultures, and slightly decreased DNA accumulation in hyoid mesenchyme cultures (Figure 17). I employed concentrations of 1.0% ethanol (for mandibular and frontonasal cultures) and 1.5% ethanol (for maxillary and hyoid cultures) in all subsequent experiments since these doses stimulated maximal or near-maximal increases in GAG production (2- to 8-fold increases) while only modestly affecting total DNA accumulation (0.8 X, 1.0 X, 1.3 X and 1.5 X control levels for hyoid, mandibular, maxillary and frontonasal cultures, respectively). In this manner, the influence of ethanol on chondrocyte differentiation could be preferentially examined while minimizing treatment effects on cell number and density.

Histochemical staining with Alcian blue confirmed that ethanol treatment induced elevations in matrix proteoglycan deposition in cultures of mesenchymal cells from all four facial primordia (Figure 16A',B',C',D') when compared to cultures maintained in control medium (Figure 16A,B,C,D). In frontonasal and mandibular process mesenchymes, which are spontaneously chondrogenic *in vitro* (Figure 16A,C), exposure to 1.0% ethanol promoted increases in cartilage matrix accumulation such that the cultures formed nearly continuous sheets of cartilage tissue that stained intensely with Alcian blue (Figure 16A',C'). In cultures of maxillary and hyoid arch

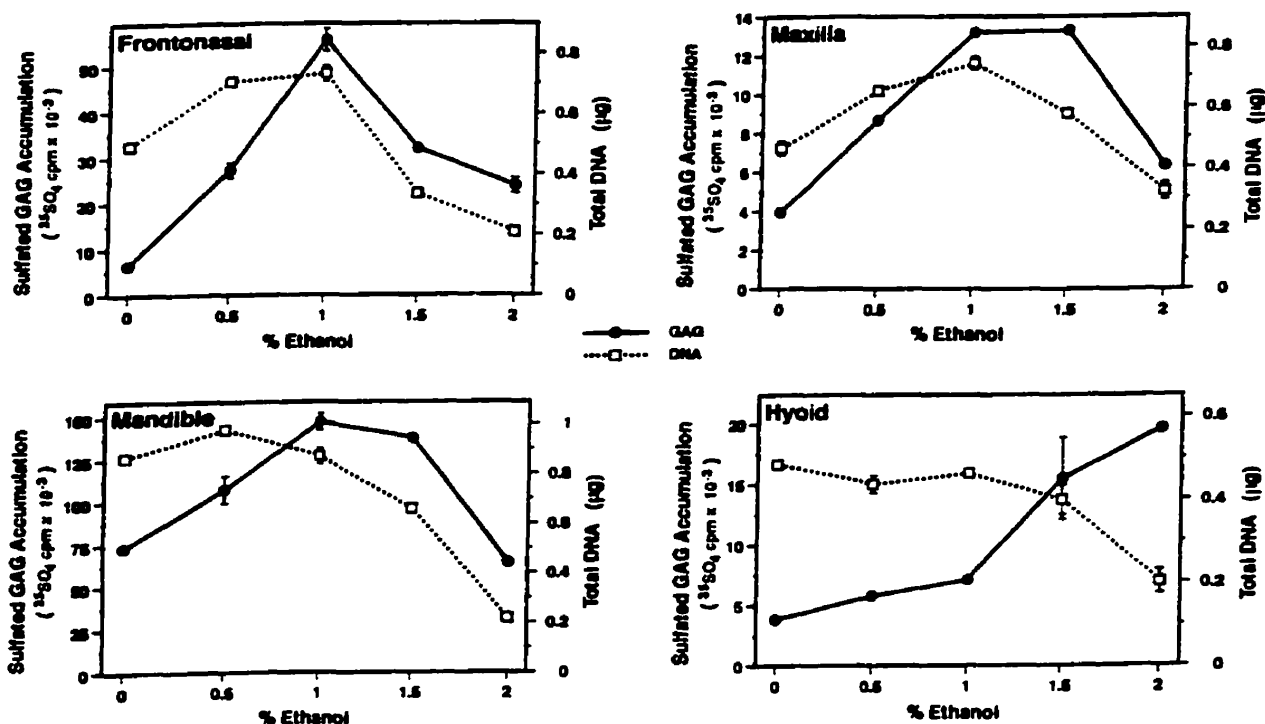


Figure 17 Effects of various concentrations of ethanol on sulfated GAG accumulation and total DNA accumulation in micromass cultures of facial mesenchyme.

Incorporation of $^{35}\text{SO}_4$ -label into total sulfated GAG (sum of cell-layer- and medium-associated GAG; closed symbols) was analyzed in cultures that were prepared from mesenchymal cells of the frontonasal, maxillary, mandibular, and hyoid processes of the stage 24 chick embryo and incubated for 3-days in medium containing 0 - 2% ethanol. Total DNA accumulation was also analyzed (open symbols). Data points represent the mean standard error of determinations from four replicate cultures. (Error bars that are not visible lie within the dimensions of the data point illustrated.)

mesenchyme, which elaborated little or no histochemically identifiable cartilage matrix when maintained in control medium (Figure 16B,D), the presence of 1.5% ethanol induced the formation of numerous Alcian blue-positive aggregates of chondrogenically differentiating cells (Figure 16B',D'). The stimulatory effect of the alcohol on matrix GAG accumulation was not simply due to a shift in the ratio of cell layer-associated versus medium-associated GAG deposition. As shown in Figure 18, ethanol treatment elevated $^{35}\text{SO}_4$ accumulation into pericellular GAG and medium-secreted GAG to similar extents.

Ethanol exposure also enhanced the expression of mRNA transcripts for type II collagen and aggrecan, the major constituents of cartilage matrix. Type II collagen and aggrecan mRNAs were undetectable in hyoid arch mesenchyme after 3 days of culture in control medium, whereas transcripts for both cartilage-characteristic genes were abundant in hyoid cultures incubated in the presence of 1.5% ethanol (Figure 19). In cultures of maxillary process mesenchyme, 1.5% ethanol treatment promoted a 5.6-fold increase in type II collagen mRNA levels (average of two independent experiments) and induced the expression of aggrecan transcripts, which were nearly undetectable in parallel control cultures (Figure 19). In cultures of the spontaneously chondrogenic frontonasal process mesenchyme, 1% ethanol treatment promoted increases of 3.4- and 3.8-fold in the steady-state levels of type II collagen and aggrecan transcripts, respectively (means of two independent experiments). Even in cultures of mandibular process mesenchyme, which exhibited the highest levels of type II collagen and aggrecan gene expression (Figure 19), treatment with 1% ethanol raised type II collagen and aggrecan mRNA expression by averages of 1.6- and 2.1-fold, respectively. (All experiments were independently repeated at least once with qualitatively equivalent results, with the exception of the initial dose response experiment shown in Figure 17. Representative data are shown, except where otherwise stated.)

3.2.2 Effects of Tertiary Butanol Treatment on Facial Mesenchyme Cultures

The stimulatory effects of alcohol on *in vitro* facial cartilage differentiation were not restricted to ethanol since, in two separate experiments, treatment with tertiary butanol caused a similar increase in chondrogenic differentiation in the facial mesenchyme cultures (Figure 20). Stimulation of chondrogenesis was optimal at 0.5% tertiary butanol (v/v) in frontonasal and mandibular mesenchyme cell cultures and at 1% tertiary butanol in maxillary and hyoid arch mesenchyme cell cultures (dose-response data not shown). At these concentrations, tertiary butanol treatment stimulated

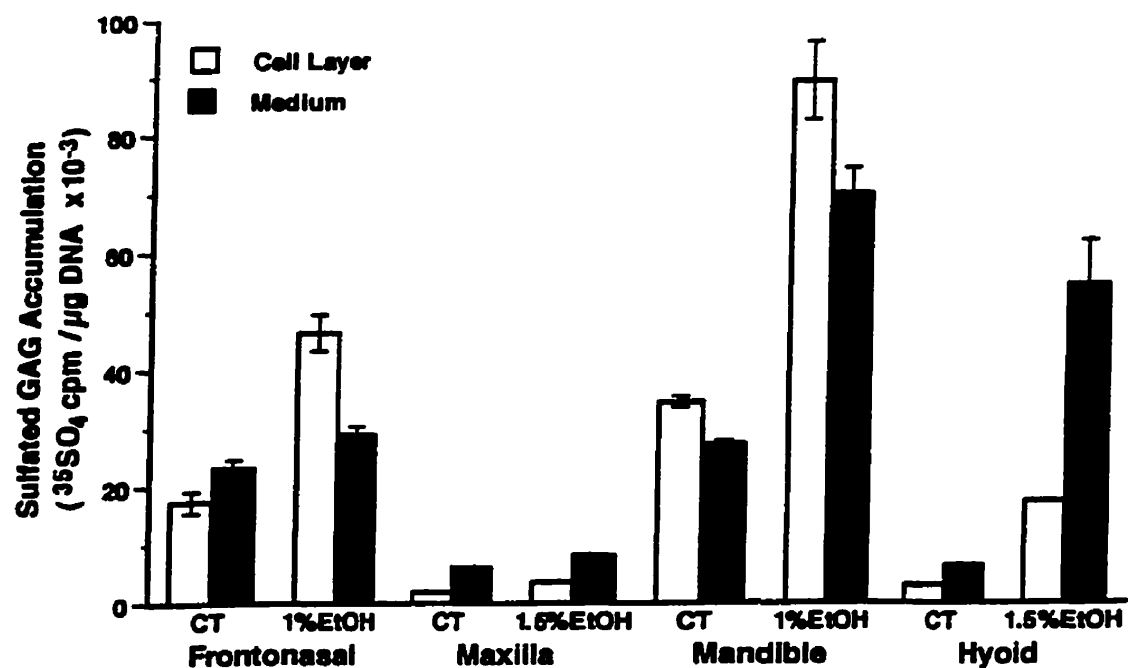


Figure 18 Sulfated GAG accumulation in ethanol-treated micromass cultures of facial mesenchyme.

Levels of $^{35}\text{SO}_4$ -labeled GAG were examined in both the cell layer and culture medium of 3-day cultures of embryonic chick facial mesenchyme cells that were incubated in the presence of 1.0 - 1.5% ethanol, or in control medium lacking ethanol. GAG accumulation is expressed per μg total cellular DNA to correct for modest differences in the DNA content of control and ethanol-treated cultures. Error bars represent the mean \pm standard deviation of determinations from four replicate cultures.

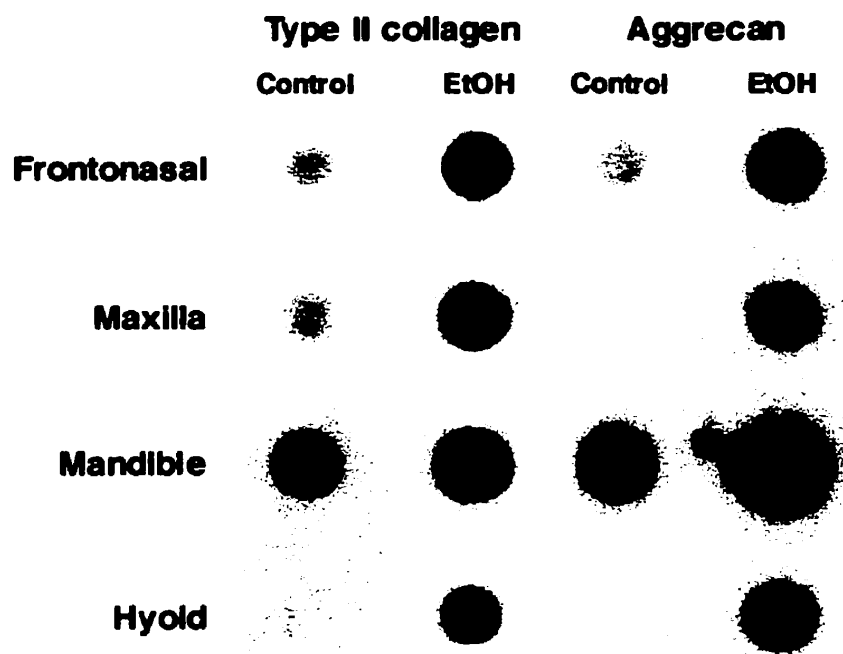


Figure 19 Dot-blot analysis examining the effects of ethanol exposure on type II collagen and aggrecan mRNA expression in micromass cultures of facial mesenchyme.

Total RNA was isolated from 3-day cultures of frontonasal, maxillary, mandibular, and hyoid process mesenchymes that were maintained in the absence (controls) or presence of 1.0 - 1.5% ethanol. All RNA samples spotted on each of the two dot-blots contained near-equivalent amounts of total poly (A)⁺ RNA as determined by hybridization with ³²P-labeled oligo (dT)₁₈.

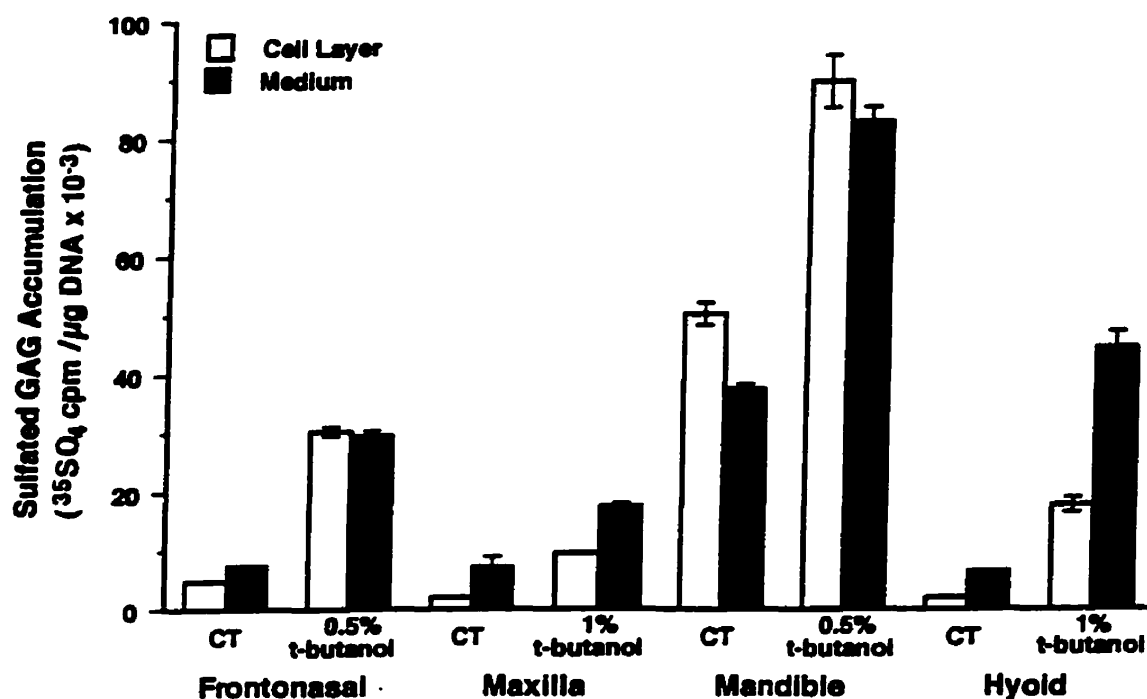


Figure 20 Sulfated GAG accumulation in micromass cultures of facial mesenchyme exposed to tertiary butanol.

Levels of $^{35}\text{SO}_4$ -labeled GAG accumulation were examined in both the cell layer and culture medium of 3-day cultures of embryonic chick facial mesenchyme cells that were incubated in the absence (CT) or presence of either 0.5% tertiary butanol (frontonasal, mandible) or 1.0% tertiary butanol (maxilla, hyoid). Error bars represent the mean \pm standard error of determinations from four replicate cultures.

significant increases in both cell layer- and medium-associated GAG accumulation within the frontonasal, maxillary, mandibular, and hyoid mesenchyme cultures (Figure 20) ($P < 0.05$; ANOVA, Bonferroni's multiple comparisons test). In addition, tertiary butanol treatment promoted increases in Alcian blue-positive matrix accumulation resembling those induced by ethanol treatment (data not shown).

3.3 *In Vitro* Effects of Ethanol Treatment on Differentiated Chondrocytes

The onset of overt chondrocyte differentiation is characterized by the activation of aggrecan gene transcript expression and a switch from type I collagen to type II collagen synthesis (Kosher et al., 1986a,b). Thereafter, proliferating chondrocytes secrete increasing amounts of extracellular matrix macromolecules until each cell is completely surrounded by an extensive matrix. The preceding results have demonstrated that ethanol potently stimulates cartilage matrix accumulation, and the expression of cartilage-specific structural genes (type II collagen and aggrecan) in cultures of chondrogenic progenitor cells isolated from embryonic limb and facial primordia. This raised the question of whether ethanol treatment selectively promotes the initial differentiation of prechondrogenic mesenchyme cells into chondroblasts, or whether it merely boosts the level of type II collagen and aggrecan gene expression in cells that have already initiated or completed chondrocyte differentiation.

Cartilage isolated from the caudal portion of the 16 - 18 day old embryonic chick sternum provides an excellent model in which to address these questions since it does not undergo endochondral ossification during embryonic and early postnatal development, and it contains an accessible population of overtly differentiated chondrocytes that express high levels of the cartilage matrix molecules, type II collagen and aggrecan (LuValle et al., 1993). Like the limb mesenchyme used in earlier experiments, sternal chondrocytes are derived from somatic mesoderm. In contrast to cartilage from the caudal portion of the chick sternum, cartilage isolated from the cranial or cephalic portion of the sternum initiates ossification changes by embryonic day 16, as evidenced by chondrocyte hypertrophy and the initiation of collagen type X gene expression (Gibson and Flint, 1985; LuValle et al., 1993). The appearance of type X collagen mRNA and protein seems to be an early event in the shift to hypertrophy, whereas a decrease in type II collagen mRNA expression and progressive matrix mineralization occur more gradually (Oshima et al., 1989).

Unlike in cultures of prechondrogenic limb and facial mesenchyme, cartilage matrix accumulation was not elevated in cultures of differentiated chondrocytes isolated

from either caudal (Figure 21A,B) or cranial (Figure 21C,D) segments of 17 - 18 day old chick embryo sterna following exposure to 1.0 - 1.5% ethanol, as evidenced by histochemical staining with Alcian blue dye and quantitative analysis of Alcian blue-positive matrix accumulation (Table 2). In three independent experiments, I observed either little effect or a modest reduction of total Alcian blue staining in ethanol-treated cultures, relative to untreated controls. Total DNA accumulation, however, was markedly reduced in both cranial and caudal sternal chondrocyte cultures following ethanol exposure (Table 2). Northern RNA analysis revealed that in cultures of both caudal and cranial sternal chondrocytes, transcript levels for type II collagen were depressed relative to controls throughout the 3 day period of incubation following ethanol exposure (Figure 22A, lanes 4-6 and 10-12). Aggrecan RNA levels were only slightly lower in ethanol-treated cultures than in controls during the first 2 days of incubation (Figure 22B, lanes 4-6 and 10-12). In addition, expression of transcripts for type X collagen, a molecular marker of chondrocyte hypertrophy, was depressed in cranial sternal chondrocytes following ethanol treatment (Figure 22C, lanes 10-12). Together, these results suggest that alcohol is not capable of exerting stimulatory effects on matrix production and cartilage-specific gene expression in chondrocytes that are already phenotypically differentiated.

3.4 Early Changes in Gene Expression Induced by Exposure of Prechondrogenic Limb Mesenchyme to Ethanol

3.4.1 Effects of Ethanol Treatment on Expression of Putative Chondrogenic Regulatory Genes

High levels of type II collagen and aggrecan mRNAs were induced in prechondrogenic limb mesenchyme cells within 24 hours of exposure to ethanol (Figure 4). In contrast, ethanol treatment did not enhance the levels of type II collagen and aggrecan mRNAs in cultures of already differentiated sternal chondrocytes (Figure 22). This suggested that ethanol might act at an early point in the chondrocyte differentiation pathway, by influencing the expression of genes involved in the initiation of chondrogenesis. Therefore, I next examined the effects of ethanol on the expression of transcripts for several genes that are preferentially expressed during the prechondrogenic cell condensation events that precede overt cartilage matrix formation in the developing limb bud and that may play regulatory roles in the activation of chondrocyte differentiation. These include *Sox-9*, Bone Morphogenetic Protein Receptor-1B (BMPR-1B), *Ets-2*, *Erg*, and TGF- β . Experiments were repeated at least

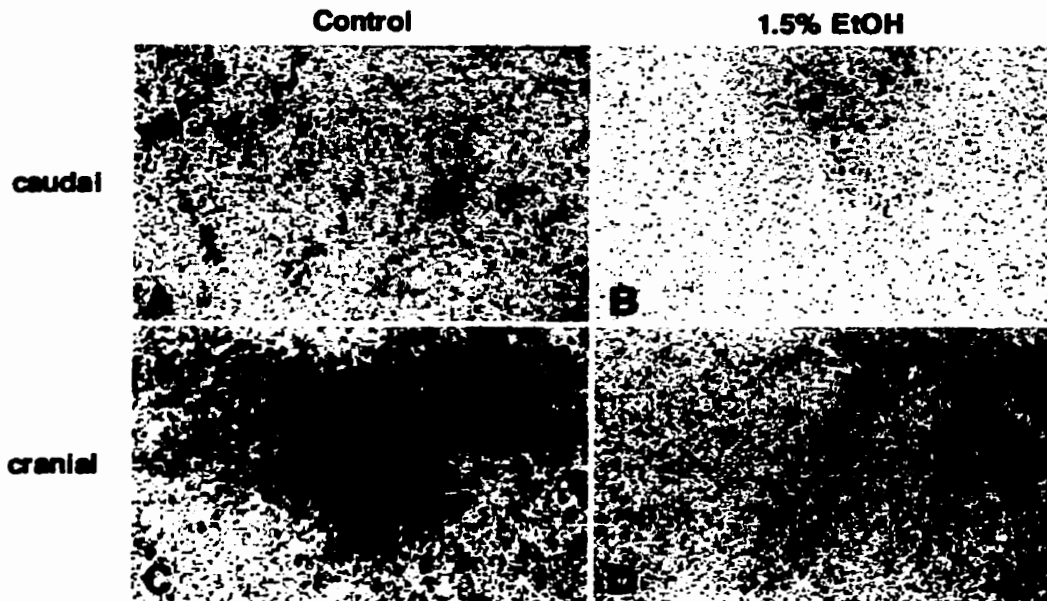


Figure 21 Photomicrographs demonstrating Alcian blue-positive matrix accumulation in ethanol-treated, monolayer cultures of differentiated sternal chondrocytes.

Chondrocytes were isolated from the caudal (**A and B**) and cranial (**C and D**) portions of 18 day old chick embryo sterna, and then cultured for 3 days in control medium (**A and C**) or in medium containing 1.5% ethanol (**B and D**). Ethanol treatment did not elevate cartilage matrix accumulation in cultures of differentiated chondrocytes. Scale bar, 1 mm for all four panels.

Table 2 Alcian blue-positive matrix accumulation in cultures of differentiated chondrocytes isolated from caudal and cranial portions of 17 day chick embryo sterna following incubation in control medium or medium supplemented with 1.0% ethanol.

	<u>Total μg Alcian Blue dye</u>	<u>Total μg DNA</u>
<u>Caudal Sternum</u>		
Control	19.97 +/- 0.31	0.62 +/- 0.02
1.0% Ethanol	12.79 +/- 0.71	0.36 +/- 0.03
<u>Cranial Sternum</u>		
Control	21.77 +/- 1.31	0.21 +/- 0.01
1.0% Ethanol	19.06 +/- 1.00	0.12 +/- 0.01

Values given indicate mean +/- standard deviation from four replicate cultures.

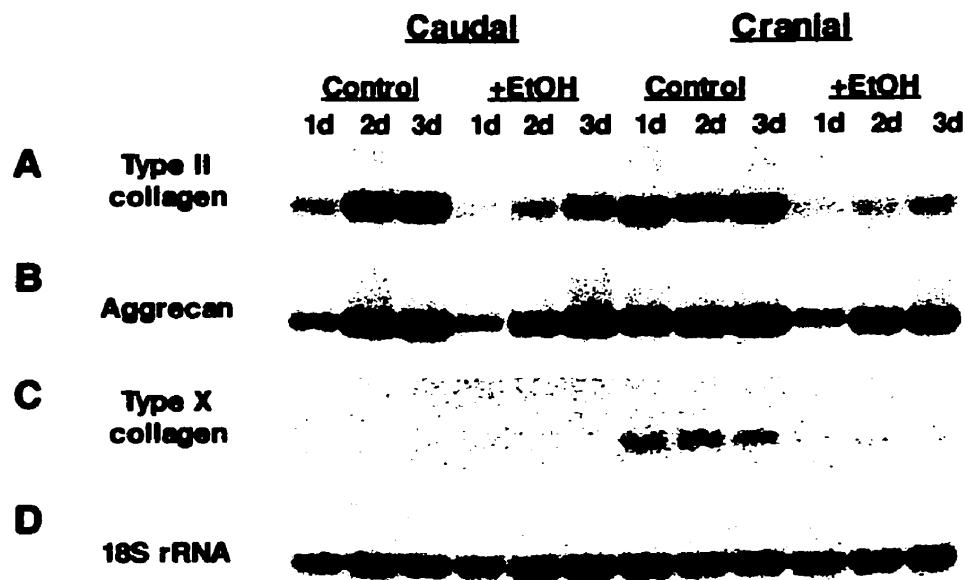


Figure 22 Northern RNA analysis examining the effects of ethanol treatment on expression of gene transcripts for type II collagen (A), aggrecan (B), and type X collagen (C) in cultures of differentiated sternal chondrocytes.

Chondrocytes were isolated from the caudal and cranial portions of 18 day chick embryos following incubation for 1 - 3 days in control medium or 1.5% ethanol. Panels A-D represent the identical Northern blot hybridized sequentially with specific cDNA probes for each transcript. All RNA samples loaded contained roughly equivalent amounts of 18S rRNA (D).

twice with equivalent results, although whole wing bud mesenchyme cultures were used in some, and distal tip mesenchyme cultures in others.

The *Sox-9* transcription factor and type II collagen are co-expressed in chondrogenic mesenchyme *in vivo* (Wright et al., 1995; Ng et al., 1997; Zhao et al., 1997; Healy et al., 1999). Evidence indicates that the appearance of *Sox-9* gene transcripts temporally precedes the upregulation of type II collagen gene expression in prechondrogenic mesenchyme cell aggregates (Ng et al., 1997; Zhao et al., 1997; Kulyk et al., 2000). Moreover, *Sox-9* is a direct transcriptional activator of the type II collagen gene (Bell et al., 1997; Lefebvre et al., 1997). Thus, *Sox-9* acts upstream of the type II collagen gene in the chondrocyte differentiation pathway. As early as 3 - 8 hours following ethanol addition, micromass cultures of distal limb mesenchyme demonstrated quantitatively greater levels of *Sox-9* mRNAs than parallel control cultures (Figure 23A). The most dramatic upregulation of *Sox-9* mRNA levels occurred at 16 - 24 hours of incubation in ethanol-treated medium. Importantly, the ethanol-induced upregulation of *Sox-9* gene expression in high-density micromass cultures of limb mesenchyme preceded any detectable upregulation of type II collagen (compare Figures 23A and 23E). In addition, ethanol treatment also elevated *Sox-9* gene expression in chondrogenically limiting, low-density cultures of stage 24/25 distal subridge mesenchyme (Figure 24A), prior to the upregulation of type II collagen RNA (Figure 24B).

Transcripts for the Bone Morphogenetic Protein Type IB Receptor (BMPR-IB) (Kawakami et al., 1996; Zou et al., 1997) and the *Ets-2* transcription factor (Maroulakou et al., 1994) have been shown to be expressed in prechondrogenic mesenchyme condensations prior to overt cartilage differentiation *in vivo*. Misexpression studies suggest that BMPR-IB activity is required for cartilage differentiation both *in vivo* and *in vitro* (Kawakami et al., 1996; Zou et al., 1997). In the present study, I demonstrated that ethanol exposure elevates both BMPR-IB (Figure 23C) and *Ets-2* (Figure 23D) gene transcript expression in high-density distal subridge mesenchyme cell cultures. The upregulation of BMPR-IB and *Ets-2* gene expression occurred approximately 16 - 24 hours following ethanol exposure, slightly prior to the activation of genes for cartilage matrix components such as type II collagen.

The *Erg* transcription factor, another member of the *Ets-2* gene family, has also been found to be expressed in cartilaginous primordia *in vivo* (Dhordain et al., 1995). However, unlike its effects on *Ets-2*, *Sox-9*, or BMPR-IB gene expression, ethanol

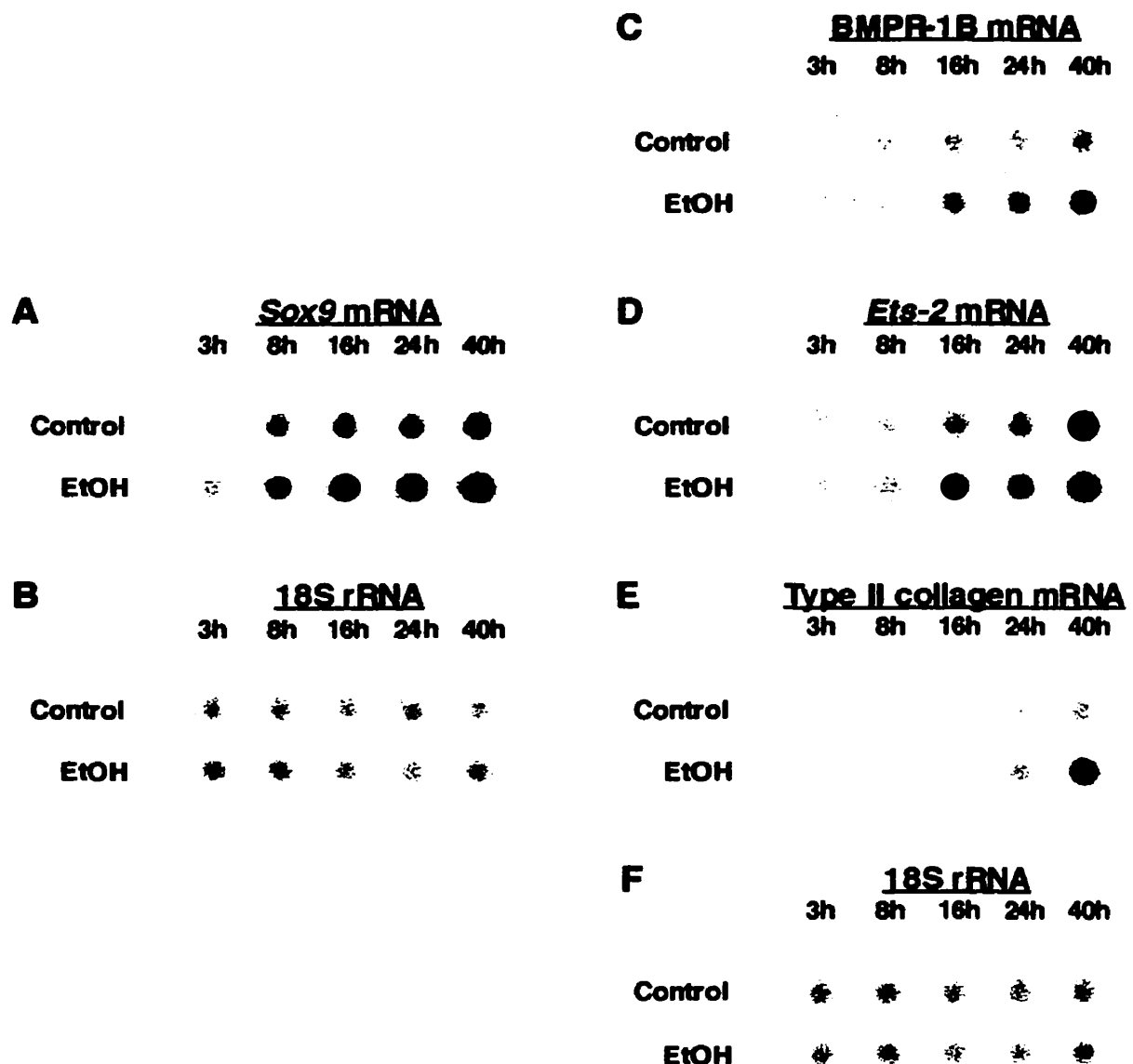


Figure 23 Dot-blot analysis examining the effects of ethanol exposure on expression of RNA transcripts for *Sox-9* (A), *BMPR-1B* (C), *Ets-2* (D), and type II collagen (E) in micromass cultures of limb mesenchyme.

Total RNA was isolated from high-density cultures of stage 24/25 distal subridge mesenchyme cells that were incubated for 3 - 40 hours in the absence (control), or presence of 1.5% ethanol. All RNA samples spotted contain roughly equivalent amounts of 18S rRNA (B and F). Note that panels A and B, and panels C-F represent two different RNA dot-blot from the same experiment that were hybridized sequentially with the above-mentioned cDNA probes.

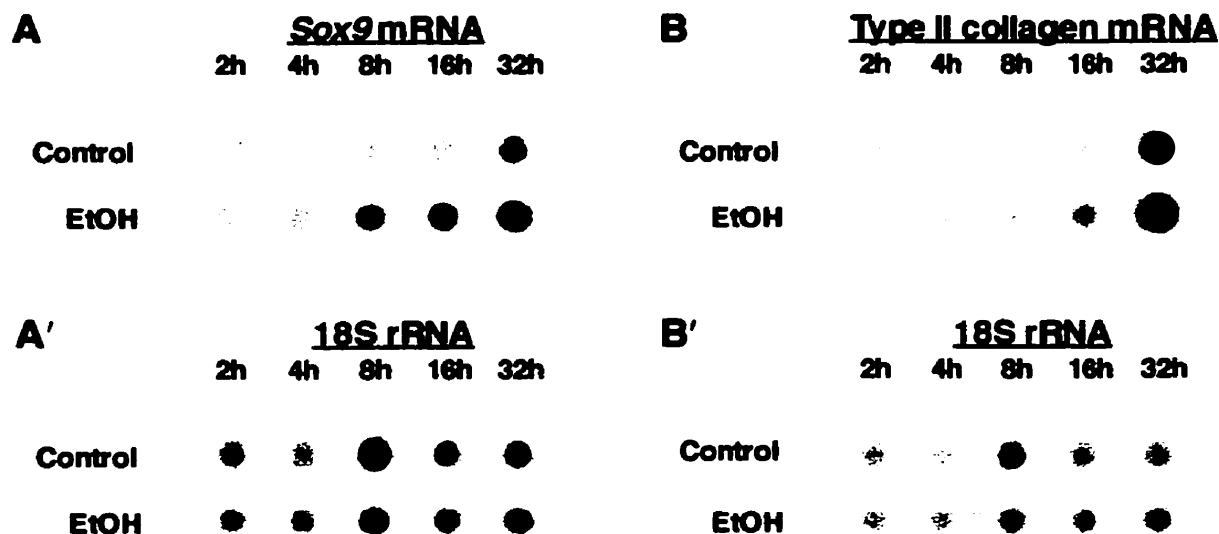


Figure 24 Dot-blot analysis examining the effects of ethanol on expression of transcripts for *Sox-9* (A), and type II collagen (B) in low-density cultures of limb mesenchyme.

Total RNA was isolated from subconfluent cultures of stage 24/25 distal subridge mesenchyme cells that were incubated for 2 - 40 hours in the absence (control), or presence of 1.5% ethanol. Each of the two dot-blots was subsequently hybridized with 18S rRNA (A' and B').

treatment had no discernible effect on the expression of *Erg* gene transcripts in high-density micromass cultures of limb mesenchyme (Figure 25A).

Peptides of the Transforming Growth Factor-Beta (TGF- β) family have been shown to promote cartilage differentiation by embryonic limb mesenchyme cells *in vitro* (Kulyk et al., 1989b; Leonard et al., 1991), and expression of TGF- β_2 , - β_3 and - β_4 RNA transcripts has been correlated with the prechondrogenic aggregation of chick limb mesenchyme cells in micromass culture (Roark and Greer, 1994). Northern blot analysis revealed that, as early as 2 - 4 hours following ethanol exposure, cultures of limb mesenchyme exhibited a dramatic depression of TGF- β_3 RNA transcripts (Figure 25B). These results provide further evidence that ethanol can induce rapid changes in gene expression in prechondrogenic mesenchyme. In contrast, I observed no obvious effect of ethanol treatment on levels of TGF- β_2 and TGF- β_4 (the chicken homologue of mammalian TGF- β_1) mRNAs in micromass culture (data not shown).

3.4.2 Effects of Ethanol Treatment on *Msx-2* Gene Expression

In the developing limb, *Msx-2* inhibits cell proliferation and promotes apoptosis in regions in which it is expressed, first in regions of anterior and posterior mesoderm that roughly demarcate the boundaries of the "progress" zone immediately subjacent to the AER, then later in regions of interdigital cell death (Coelho et al., 1991; Ferrari et al., 1998). Thus, *Msx-2* may act to restrict skeletogenesis to the center or core of the developing limb and digits. Recently, it has been shown that ethanol treatment not only inhibits the expression of *Msx-2* gene transcripts in cultured calvarial osteoblasts, but also completely abolishes *Msx-2* RNA expression in developing mouse embryos *in vivo* (Rifas et al., 1997). As such, it was suggested that inhibition of *Msx-2* expression might be the pathogenic basis of fetal alcohol effects (Rifas et al., 1997). For this reason, I examined whether ethanol might stimulate cartilage differentiation in limb mesenchyme cultures by inhibiting *Msx-2* expression. In three separate experiments, however, I observed that ethanol treatment had no effect on the expression of *Msx-2* gene transcripts in micromass cultures of prechondrogenic limb mesenchyme (Figure 25C).

3.4.3 Effects of Ethanol Treatment on Expression of Hsp Stress Response Genes

Studies in other cell types indicate that a stress response involving the activation of Hsp 70 and other heat-shock genes may be elicited when cells are exposed to alcohol (Li, 1983; Hahn et al., 1985; Neuhaus-Steinmetz et al., 1993). To determine whether the stimulatory effects of ethanol on cartilage differentiation are secondary to induction

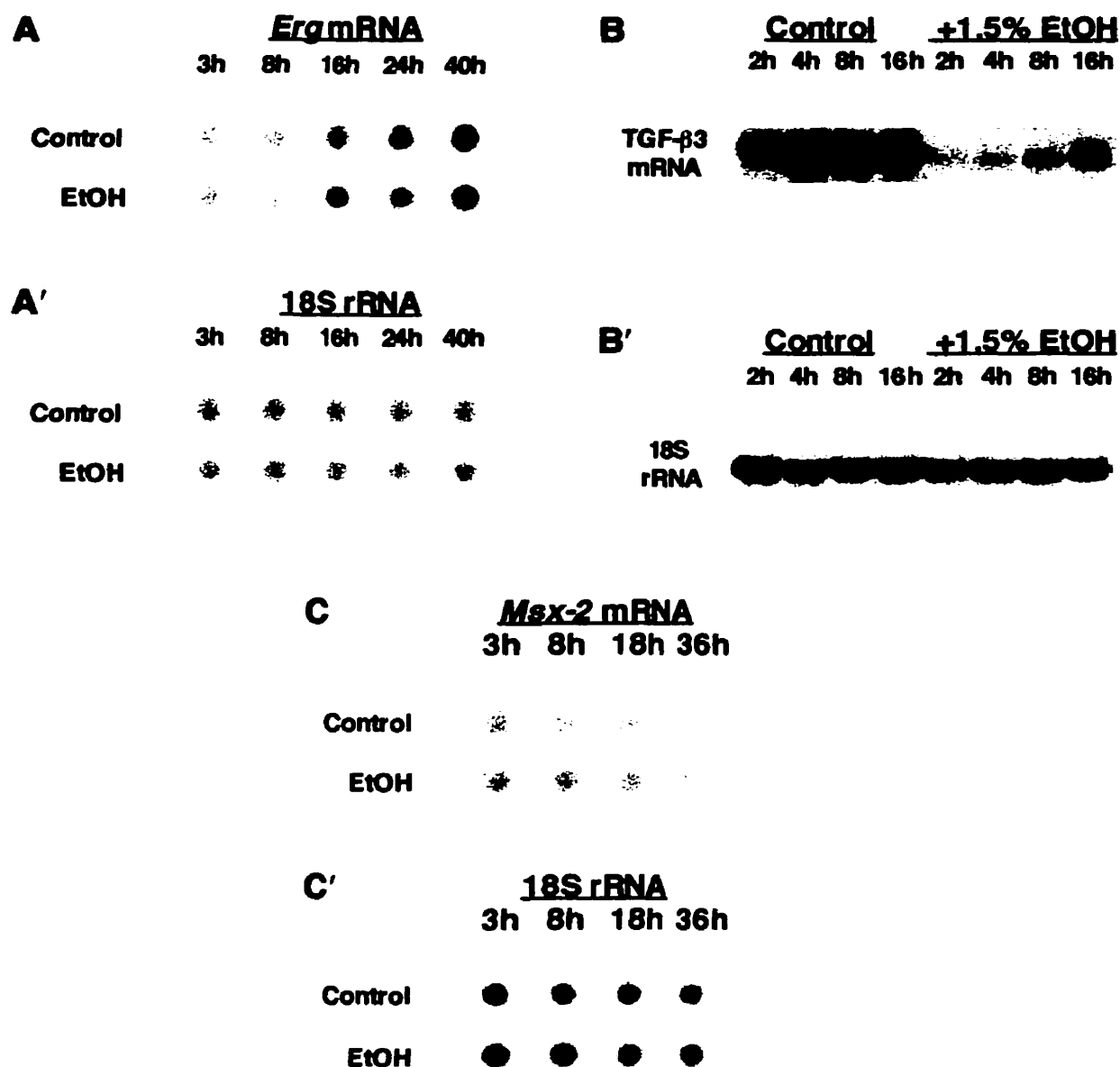


Figure 25 Effects of ethanol exposure on *Erg*, TGF- β ₃ and *Msx-2* gene expression in micromass cultures of limb bud mesenchyme.

RNA dot-blot analyses (A, A', C, C') or Northern blot analysis (B, B') demonstrate relative levels of RNA transcripts for *Erg* (A), TGF- β ₃ (B), and *Msx-2* (C) in high-density micromass cultures of stage 23/24 whole wing bud mesenchyme (C, C'), and stage 24/25 distal subridge mesenchyme (A, A', B, B') that were incubated for 2 - 40 hours in the absence (control) or presence of 1.5% ethanol. For each probe, all RNA samples spotted contain roughly equivalent amounts of 18S rRNA (A', B', C').

of a classic stress response, I examined the expression of transcripts for the Hsp70, Hsp47, and Hsp90 α stress-response genes in response to 2% ethanol. Micromass cultures of whole wing bud mesenchyme were established as previously described, and incubated overnight in untreated control medium. On the following day, certain cultures were exposed to 2% ethanol, or heat-shocked at 45°C for 0 - 6 hours.

Little or no Hsp70 RNA expression was discernible in micromass cultures of limb mesenchyme after 0.5 to 6 hours of ethanol exposure, or an equivalent period of incubation in control medium (37°C). In contrast, a 45°C heat-shock rapidly induced Hsp 70 expression (Figure 26A).

There was a low level expression of transcripts for the Hsp47 gene in control cultures of limb mesenchyme, with a notable rise in expression at later time points of incubation. As expected, Hsp47 gene expression was markedly elevated in cultures that were subjected to a 45°C heat-shock for 2-6 hours. In contrast, ethanol-treated cultures demonstrated Hsp47 mRNA levels that were only modestly higher than those of controls after 4 - 6 hours incubation (Figure 26B).

Hsp90 α mRNA expression was also strongly induced in limb mesenchyme cultures that had been heat-shocked, but was barely discernible in control cultures until 4 - 6 hour time points. Ethanol-treated cultures exhibited only slightly elevated levels of Hsp90 α mRNAs relative to controls (Figure 26C).

Together, these results suggest that ethanol concentrations that are effective in stimulating *in vitro* chondrogenesis do not elicit a significant classic stress response in prechondrogenic limb mesenchyme cells.

3.4.4 "Differential Display": A Technique to Identify Novel Genes that are Preferentially Expressed During Ethanol-Induced Chondrocyte Differentiation

The "Differential Display" PCR cloning method (Liang and Pardee, 1992; Aiello et al., 1994; Nishio et al., 1994) was used in an attempt to identify early response genes that might be preferentially expressed during ethanol-induced chondrocyte differentiation. In theory, the Differential Display method allows one to identify a subset of genes that are differentially expressed in two cell populations (e.g., ethanol-treated vs. control) without any prior knowledge of the nature or sequence of the genes. Briefly, reverse transcriptase PCR (RT-PCR) is performed using various combinations of arbitrary and poly A⁺ tail-anchored oligonucleotide primers in order to generate a set of cDNA fragments derived from total mRNA of each cell population. The PCR fragments derived from the two cell populations are then separated on high-

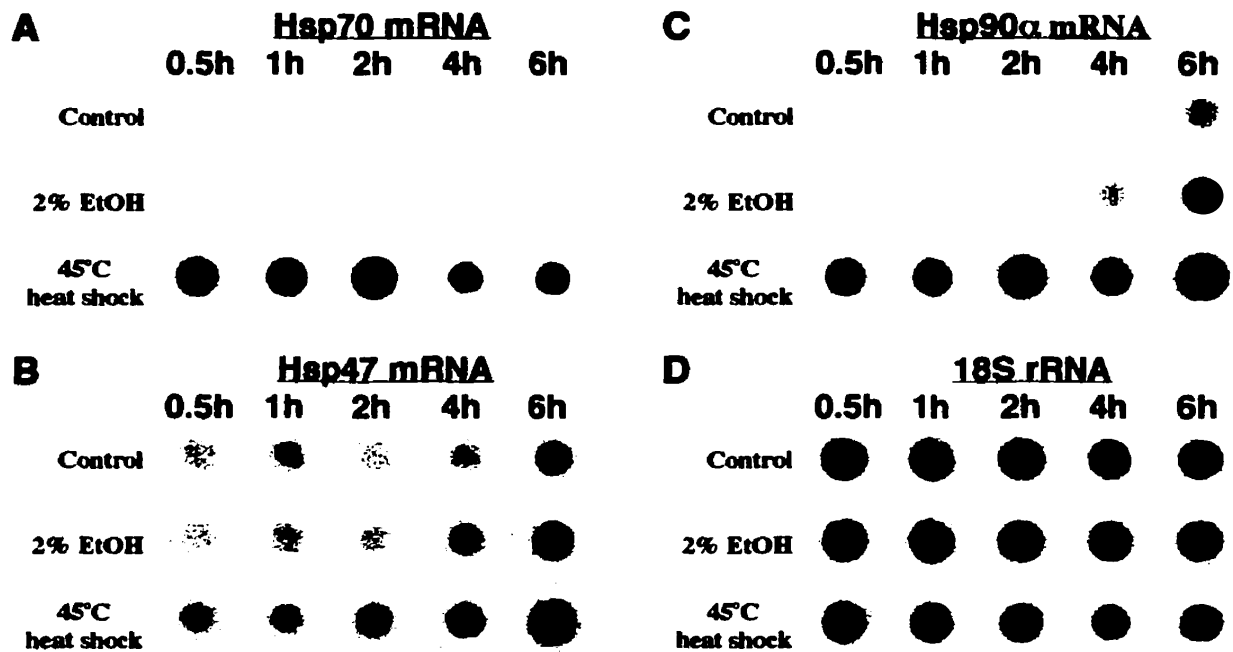


Figure 26 Dot-blot analysis examining the effects of ethanol treatment on gene transcript expression of Hsp70 (A), Hsp47 (B), and Hsp90α (C) in micromass cultures of stage 23/24 whole wing bud mesenchyme.

Total RNA was isolated from cultures that were incubated for 0.5 - 6 hours in the absence (control) or presence of 2% ethanol, or following a 45°C heat shock. Panels A-D represent the identical RNA dot-blot hybridized sequentially with specific cDNA probes for each transcript.

resolution sequencing gels to identify transcripts that are selectively up- or downregulated. The method is theoretically capable of generating reproducible patterns of bands that represent almost all expressed genes in a particular cell.

In the present study, low-density cultures of distal subridge limb mesenchyme, which undergo relatively little spontaneous cartilage formation, were incubated in the presence or absence (untreated control) of 1.5% ethanol. I presumed that ethanol might rapidly activate genes that wouldn't normally be expressed under these chondrogenically-limiting conditions. Following an 18 hour incubation in control or ethanol-treated medium, RNA was isolated from each cell population, and RT-PCR carried out using differential display primers. The 18 hour time point was chosen since it was the earliest point at which 1.5% ethanol was observed to significantly enhance the levels of type II collagen and aggrecan mRNAs in low-density cultures of prechondrogenic limb mesenchyme (data not shown). As shown in Figure 27A, when the amplified cDNA fragments were electrophoretically separated on 6% non-denaturing polyacrylamide gels, a number of candidate "differentially expressed" sequences were identifiable as discrete bands. Approximately 25 PCR fragments appeared to be strongly up- or downregulated in ethanol-treated cells, and were subsequently eluted from the gel, reamplified, and cloned into a T/A vector. However, subsequent RNA dot-blot analysis, using the cloned fragments of interest (e.g., D6U9) as probes, failed to confirm that any of the sequences I had isolated were truly differentially expressed in control and ethanol-treated cultures of prechondrogenic limb mesenchyme plated at subconfluent density (Figure 27B). What initially appeared to be unique bands after gel purification of the reamplified product may have contained a heterogeneous mixture of unresolved cDNAs (false positives). The dot-blots against which my candidate PCR fragments were screened showed a typical response to ethanol treatment since transcript levels for aggrecan were dramatically upregulated in low-density cultures of limb mesenchyme 18 hours following exposure to 1.5% ethanol (Figure 27C). Due to time constraints, I was forced to discontinue this line of experiments.

3.5 Role of Specific Intracellular Signal Transduction Pathways in Mediating Ethanol's Effects on Chondrogenesis

Studies in other cell types have demonstrated that alcohols can perturb a number of intracellular signal transduction pathways involved in cell-cell signaling and metabolic control (Taraschi and Rubin, 1985; Littleton, 1989; Hoek and Rubin, 1990). In an attempt to examine the mechanisms through which ethanol exerts its

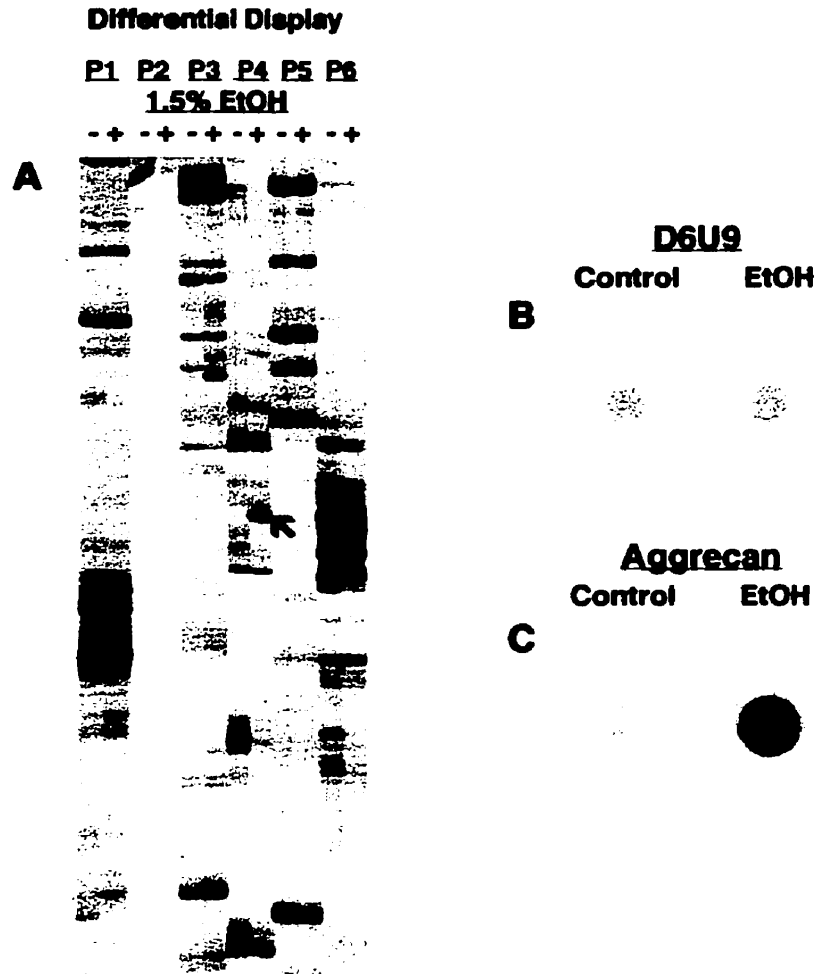


Figure 27 Identification of genes differentially expressed at the mRNA level using a novel PCR-based selective cloning strategy, "Differential Display".

(A) RT-PCR was performed using various pairs of arbitrary and poly A+ tail-anchored oligonucleotide primers (e.g., P1-P6) in order to generate a set of cDNA fragments derived from total mRNA of control (-) and ethanol-treated (+) limb mesenchyme cells. The resulting PCR fragments were then separated on high-resolution sequencing gels to identify transcripts that are selectively up- or downregulated. (B) The amplified D6U9 sequence indicated by the arrow in panel A was excised from the gel, cloned into pCRII vector, and then used to probe a dot-blot of total RNA from low-density cultures of limb mesenchyme cells that had been cultured for 18 hours in either control medium or 1.5% ethanol. This screening revealed that transcripts recognized by the cloned D6U9 sequence were not truly differentially expressed. (C) In contrast, hybridization of the same RNA samples with an aggrecan cDNA probe verified that the 1.5% ethanol treatment had induced a prominent increase in cartilage-specific gene expression.

stimulatory effects on chondrogenesis, I employed a variety of pharmacological agents that inhibit specific intracellular kinase pathways. Cultures were then co-treated with ethanol to determine whether ethanol's ability to enhance cartilage differentiation is prevented following inhibition of a particular pathway. Micromass cultures of whole wing bud mesenchyme were plated at high density ($1 - 2 \times 10^5$ cells/10 μ l), as previously described, and initially fed with either control medium or medium containing a specific pharmacological inhibitor. The particular inhibitor doses employed were selected based on preliminary dose-response experiments conducted in our laboratory, and on published K_i constants (inhibition constants) or IC_{50} values (concentration required to produce 50% inhibition). A concentration was used that met or exceeded the dose reported to selectively inhibit a specific signaling pathway, but was low enough to prevent nonspecific inhibition of other signaling pathways, and was not blatantly cytotoxic. Following a 12 hour pre-incubation with the inhibitor alone, cultures were incubated for an additional 48 hours in the presence of either the inhibitor alone, or both inhibitor and 1.5% ethanol.

3.5.1 Protein Kinase C (PKC) Inhibitors

After 3 days of incubation, micromass cultures incubated in untreated control medium developed numerous Alcian blue-positive cartilage nodules (Figure 28A). A similar pattern was seen when the prechondrogenic mesenchyme cells were cultured in the presence of various pharmacological PKC inhibitors, including 0.5 μ M Bisindolylmaleimide I (Figure 28B), 1 μ M Chelerythrine (Figure 28C), and 25 μ M H-7 (Figure 28D). Bisindolylmaleimide has been shown to be a highly selective, cell permeable PKC inhibitor ($K_i = 10$ nM)(Toullec et al., 1991; Kiss et al., 1995). Chelerythrine is also a potent and selective inhibitor of PKC ($IC_{50} = 660$ nM)(Herbert et al., 1990; von Stebut et al., 1994). H-7, on the other hand, is a relatively broad-spectrum, cell permeable ser/thr kinase inhibitor, inhibiting not only PKC ($K_i = 6$ μ M), but also myosin light chain kinase ($K_i = 97$ μ M), PKA ($K_i = 3$ μ M), and PKG ($K_i = 5.8$ μ M) (Hidaka et al., 1984; Quick et al., 1992). Cultures of limb mesenchyme cells that were incubated in the presence of ethanol alone following a 12 hour pre-incubation in control medium (Figure 28A') exhibited considerably greater cartilage matrix deposition than untreated control cultures (Figure 28A). Parallel cultures treated with 1.5% ethanol at the time of initial feeding elaborated still further cartilage matrix (data not shown). The presence of Bisindolylmaleimide, Chelerythrine, or H-7 did not block the ability of ethanol to elevate Alcian blue-positive matrix accumulation (compare Figures

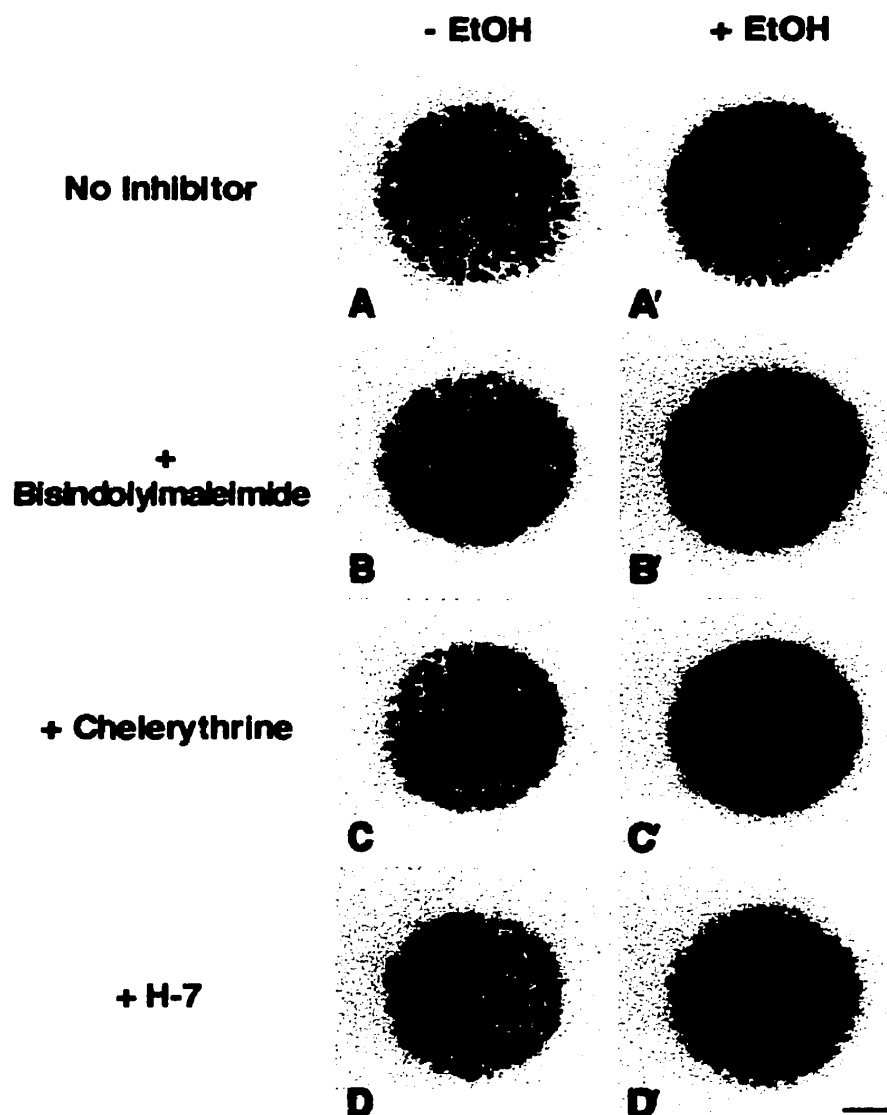


Figure 28 Photomicrographs demonstrating effects of various Protein Kinase C (PKC) inhibitors on ability of ethanol to enhance Alcian blue-positive cartilage matrix accumulation *in vitro*.

Micromass cultures of stage 23/24 whole wing bud mesenchyme were plated at 2×10^5 cells/ $10\mu\text{l}$ medium. Cultures were subsequently stained with Alcian blue, pH 1, following 3 days of incubation in control medium (A); 3 days in medium containing $0.5 \mu\text{M}$ Bisindolylmaleimide alone (B), $1 \mu\text{M}$ Chelerythrine alone (C) or $25 \mu\text{M}$ H-7 alone (D); a 12 hour preincubation in control medium followed by 48 hours in 1.5% ethanol (A'); or a 12 hour preincubation with each of the inhibitors alone, followed by 48 hours in the presence of both inhibitor and 1.5% ethanol (B', C', D'). Scale bar, 1 mm for all photographs.

28B',C',D' to Figures 28B,C,D), suggesting that ethanol does not appear to require an active PKC signaling pathway to stimulate *in vitro* chondrogenesis.

Quantitative analysis confirmed that none of the PKC inhibitors alone had any significant effect on cartilage matrix accumulation/ μ g DNA, relative to untreated control cultures (Figure 29) ($P > 0.05$; ANOVA, Tukey's multiple comparisons test). Moreover, even in the presence of these inhibitors, ethanol still exerted significant stimulatory effects on chondrogenic differentiation *in vitro*, relative to cultures treated with each of the inhibitors alone (Figure 29) (significant at $P < 0.001$). (The experiments with Chelerythrine and H-7 were each performed once, whereas the effects of Bisindolylmaleimide were examined in three independent experiments with equivalent results.)

3.5.2 PKC Downregulators

Prolonged exposure to PKC-activating phorbol esters (e.g., PMA), as well as to certain non-phorbol PKC activators (e.g., Mezerein and OIL), causes a proteolytic depletion or "downregulation" of intracellular PKC (Rodriquez-Pena and Rozengurt, 1984; Ballester and Rosen, 1985; Fournier and Murray, 1987; Sonn and Solursh, 1993; Jia, 1995). Therefore, PKC signaling can be experimentally blocked by chronic treatment with these agents. Micromass limb mesenchyme cell cultures that were incubated for 3 days in medium containing either 100 nM PMA (Figure 30B), 30 nM Mezerein (Figure 30C), or 25 nM OIL (Figure 30D) elaborated considerably less Alcian blue-positive cartilage matrix than parallel untreated control cultures (Figure 30A). The pronounced reduction of Alcian blue staining in these cultures appears to be partially due to a reduction of total cellular DNA content (Figure 30). Quantitative analysis of Alcian blue matrix accumulation/ μ g DNA, however, revealed that each of these agents alone inhibited chondrogenesis relative to untreated controls (Figure 31) (significant at $P < 0.001$). When PMA-, Mezerein- and OIL-treated cultures were cotreated with ethanol, there was a statistically significant increase in matrix formation ($P < 0.001$), although it did not recover to the level observed in untreated control cultures, and certainly not to the level observed in ethanol-treated cultures (Figures 30B',C',D' and Figure 31). (Experiments with Mezerein and OIL were each performed once; the effects of PMA were examined in 3 separate experiments with equivalent results.)

RNA dot-blot analysis (Figure 32) supports the above findings. In two independent experiments, micromass cultures of stage 23/24 whole wing bud mesenchyme that were incubated in the presence of 1.5% ethanol (Figure 32A,B, lanes 2 and 3) demonstrated higher levels of both type II collagen (Figure 32A) and *Sox-9*

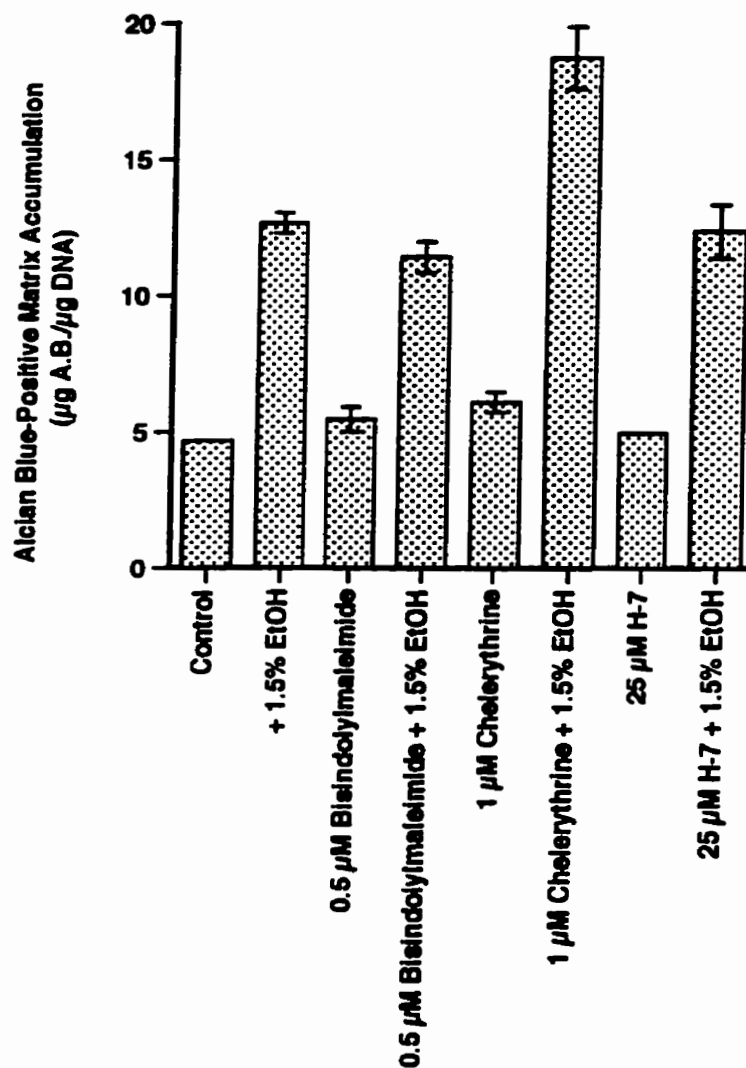


Figure 29 Graph illustrating effects of various PKC inhibitors on ability of ethanol to enhance sulfated GAG accumulation in micromass cultures of whole wing bud mesenchyme.

Levels of Alcian blue-positive matrix accumulation/µg DNA were examined in micromass cultures prepared from stage 23/24 whole wing bud mesenchyme, and incubated for 3 days in control medium; 3 days in medium containing 0.5 µM Bisindolylmaleimide alone, 1 µM Chelerythrine alone or 25 µM H-7 alone; a 12 hour preincubation in control medium, followed by 48 hours in 1.5% ethanol; or a 12 hour preincubation with each of the inhibitors alone, followed by 48 hours in the presence of both inhibitor and 1.5% ethanol. Bars represent the mean +/- standard deviation of measurements from four replicate cultures.

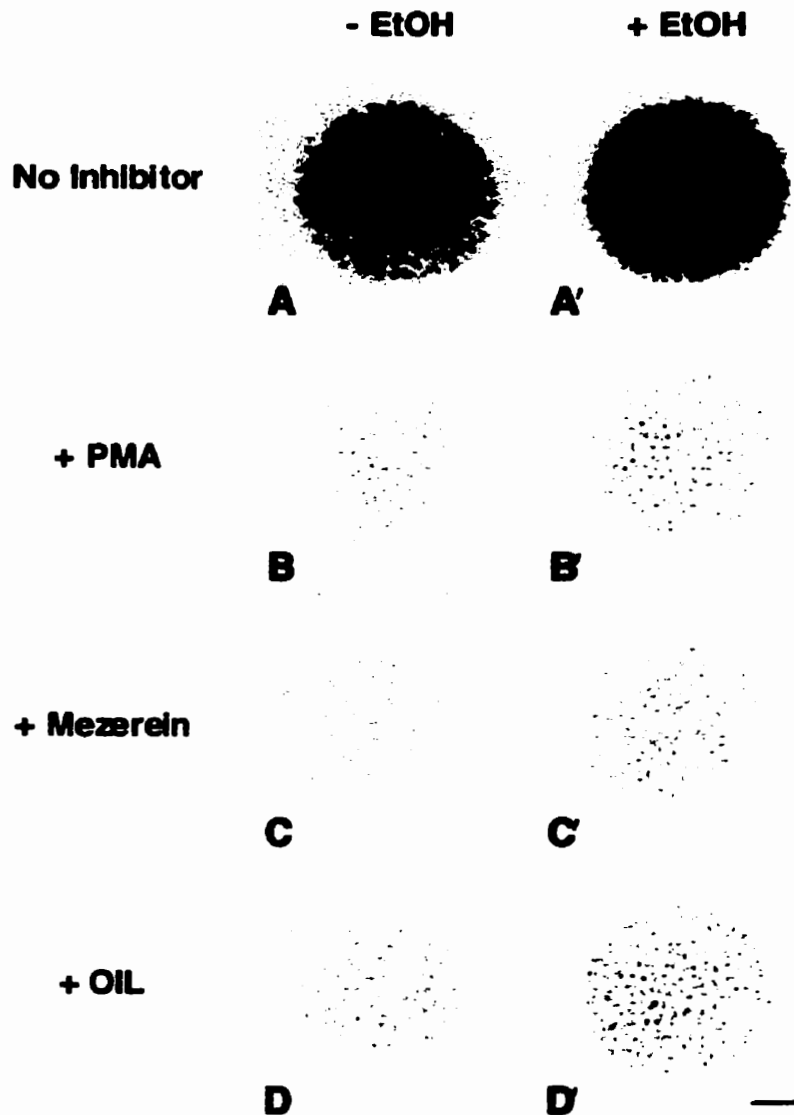


Figure 30 Photomicrographs illustrating the influence of various PKC downregulators on ability of ethanol to enhance Alcian blue-positive cartilage matrix accumulation *in vitro*.

Micromass cultures of stage 23/24 whole wing bud mesenchyme were plated at 2×10^5 cells/ $10\mu\text{l}$ medium. Cultures were then stained with Alcian blue, pH 1, following 3 days of incubation in control medium (A); 3 days in medium containing 100 nM PMA alone (B), 30 nM Mezerein alone (C) or 25 nM OIL alone (D); a 12 hour preincubation in control medium followed by 48 hours in 1.5% ethanol (A'); or a 12 hour preincubation with each of the inhibitors alone, followed by 48 hours in the presence of both inhibitor and 1.5% ethanol (B', C', D'). Scale bar, 1 mm.

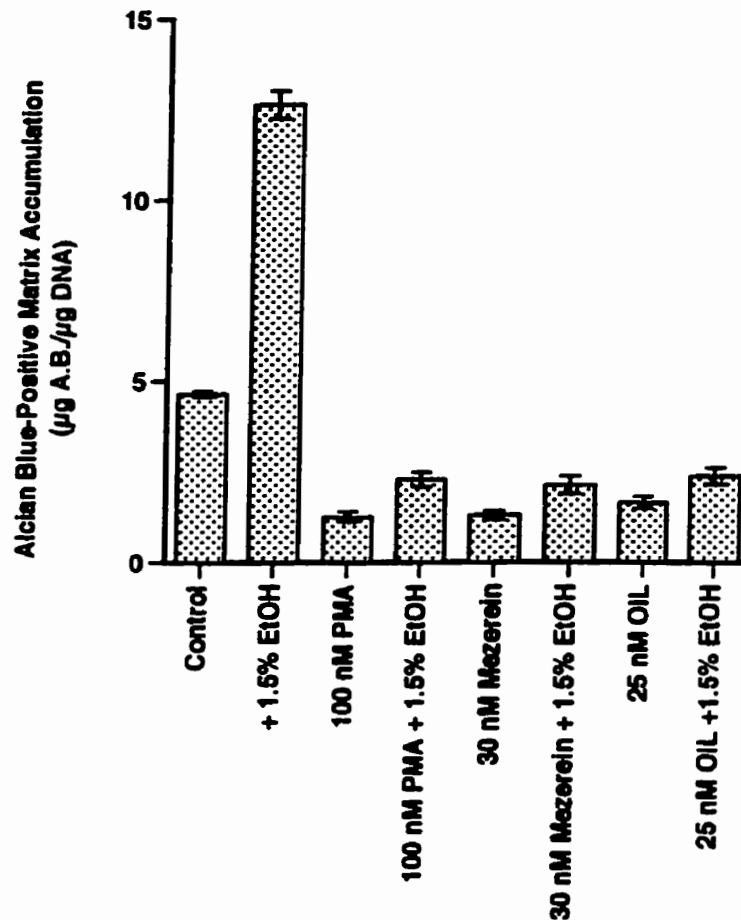


Figure 31 Graph demonstrating effects of various PKC downregulators on ability of ethanol to enhance sulfated GAG accumulation in micromass cultures of whole wing bud mesenchyme.

Levels of Alcian blue-positive matrix accumulation/ μ g DNA were examined in high-density cultures prepared from stage 23/24 whole wing bud mesenchyme, and incubated for 3 days in control medium; 3 days in medium containing 100 nM PMA alone, 30 nM Mezerein alone or 25 nM OIL alone; a 12 hour preincubation in control medium, followed by 48 hours in 1.5% ethanol; or a 12 hour preincubation with each of the inhibitors alone, followed by 48 hours in the presence of both inhibitor and 1.5% ethanol. Bars represent the mean \pm standard deviation of measurements from four replicate cultures.

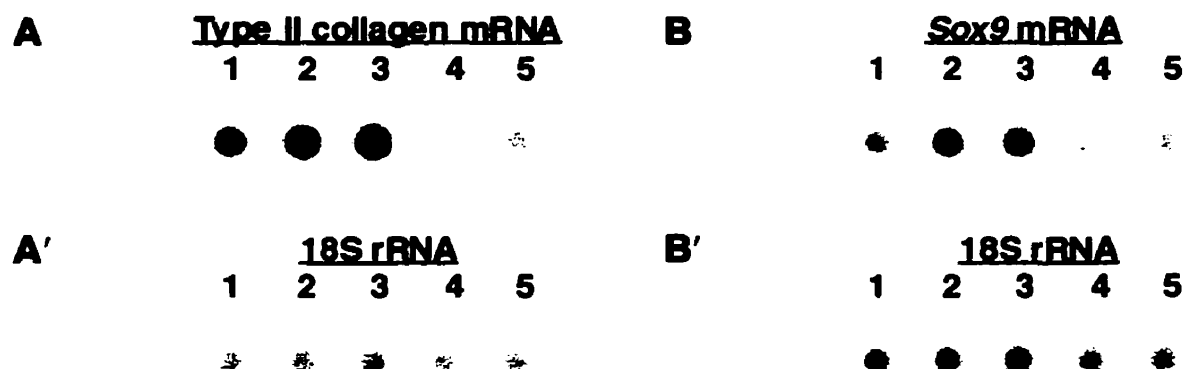


Figure 32 Effects of PMA on ability of ethanol to enhance type II collagen and *Sox-9* gene expression in micromass cultures of limb bud mesenchyme.

RNA dot blot analysis demonstrates relative levels of gene transcripts for type II collagen (**A**), and *Sox-9* (**B**) in micromass cultures of stage 23/24 whole wing bud mesenchyme incubated for 3 days in control medium (**lane 1**); 3 days in medium supplemented immediately with 1.5% ethanol (**lane 3**); 3 days in medium supplemented with 100 nM PMA alone (**lane 4**); a 12 hour preincubation in control medium, followed by 48 hours in 1.5% ethanol (**lane 2**); or a 12 hour preincubation in medium containing 100 nM PMA, followed by 48 hours in the presence of both 100 nM and 1.5% ethanol (**lane 5**). For each probe, all RNA samples contain roughly equivalent amounts of 18S rRNA (**A'**, **B'**).

(Figure 32B) mRNAs following a 3 day incubation period, compared to parallel untreated control cultures (Figure 32A,B, lane 1). In contrast, expression of type II collagen (Figure 32A) and *Sox-9* (Figure 32B) gene transcripts was markedly reduced in cultures that were exposed continuously to 100 nM PMA (Figure 32A,B, lane 4). When mesenchyme cell cultures were cotreated with both PMA and ethanol (Figure 32A,B, lane 5), both type II collagen and *Sox-9* gene expression were enhanced relative to cultures fed with PMA alone (Figure 32A,B, lane 4). Levels of *Sox-9* gene expression in the cotreated cultures approached those levels observed in control cultures, whereas levels of type II collagen RNA did not (compare lanes 1 and 5 of Figure 32A,B). However, although ethanol allowed a partial recovery of matrix accumulation and cartilage-specific gene expression (type II collagen and *Sox-9*) in the presence of PKC downregulators, these levels were much lower than with ethanol alone.

Together, these results suggest that the downregulation of PKC by PMA, Mezerein, and OIL greatly reduces, but does not completely abolish the ability of ethanol to stimulate chondrogenesis.

3.5.3 Mitogen-Activated Protein (MAP) Kinase Inhibitors

Treatment of cultured prechondrogenic mesenchyme cells with 10 μ M PD98059, an inhibitor of the ERK (p42/44 MAP kinase) pathway resulted in a greater than 2-fold stimulation of Alcian blue-stainable matrix formation per μ g DNA (Figure 33B and Figure 34) relative to untreated control cultures (Figure 33A and Figure 34) ($P < 0.001$; ANOVA, Tukey's multiple comparisons test). PD98059 has been shown to selectively block the activity of MAP kinase kinase (MEK), which phosphorylates and activates ERK ($IC_{50} = 2 \mu$ M)(Alessi et al., 1995; Dudley et al., 1995). Cultures cotreated with both PD98059 and ethanol exhibited even higher levels of cartilage formation than those treated with PD98059 alone (Figure 33B' and Figure 34) (significant at $P < 0.001$). This study was repeated twice with equivalent results, suggesting that active ERK pathway signaling is not required for ethanol to stimulate *in vitro* chondrogenesis.

Exposure of cells to 1 μ M Wortmannin produced an enhancement of chondrogenic differentiation (Figure 35B and Figure 36) ($P < 0.001$) relative to untreated control cultures (Figure 35A) that was qualitatively similar to that of PD98059. Wortmannin acts a potent, cell permeable and irreversible inhibitor of PI-3 kinase ($IC_{50} = 5 \text{ nm}$), although it also inhibits signaling through the ERK MAP kinase pathway at concentrations greater than 200-300 nM (Ferby et al., 1994; Powis et al., 1994; Duckworth and Cantley, 1997). Like PD98059, cultures that were co-treated

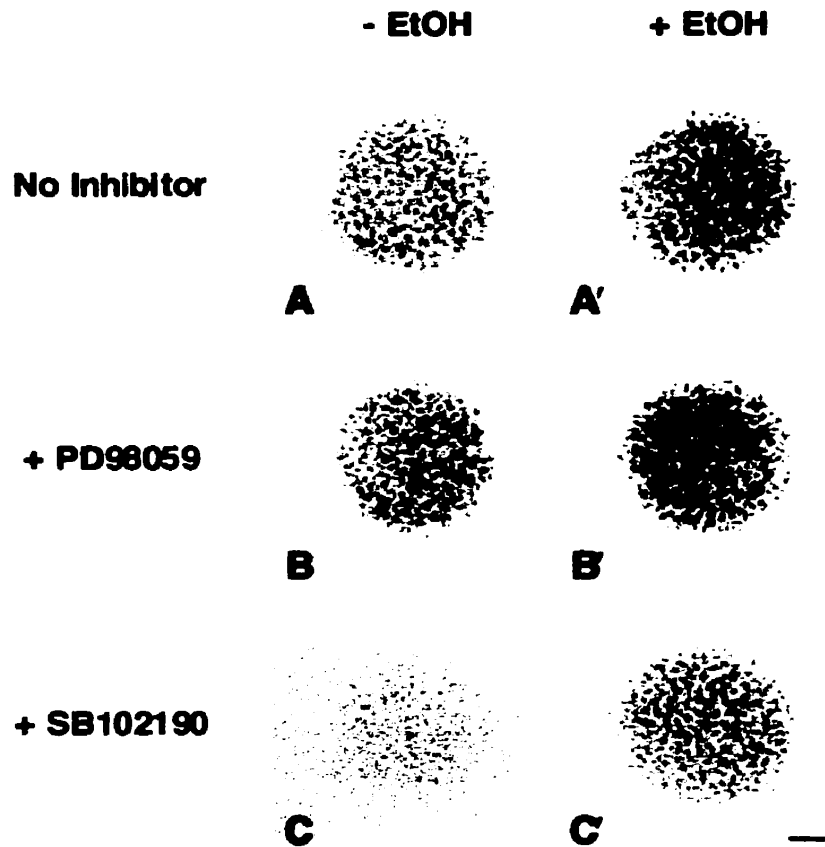


Figure 33 Photomicrographs illustrating the influence of various Mitogen-Activated Protein (MAP) Kinase inhibitors on ability of ethanol to enhance Alcian blue-positive cartilage matrix accumulation *in vitro*.

Micromass cultures of mesenchyme cells were established from whole stage 23/24 wing buds and plated at 1×10^5 cells/ $10\mu\text{l}$ medium. Cultures were stained with Alcian blue, pH 1, following 3 days of incubation in control medium (A); 3 days in medium containing $10\mu\text{M}$ PD98059 (an inhibitor of the ERK MAP kinase pathway) alone (B) or $1\mu\text{M}$ SB202190 (an inhibitor of the p38 MAP kinase pathway) alone (C); a 12 hour preincubation in control medium followed by 48 hours in 1.5% ethanol (A'); or a 12 hour preincubation with each of the inhibitors alone, followed by 48 hours in the presence of both inhibitor and 1.5% ethanol (B', C'). Scale bar, 1 mm.

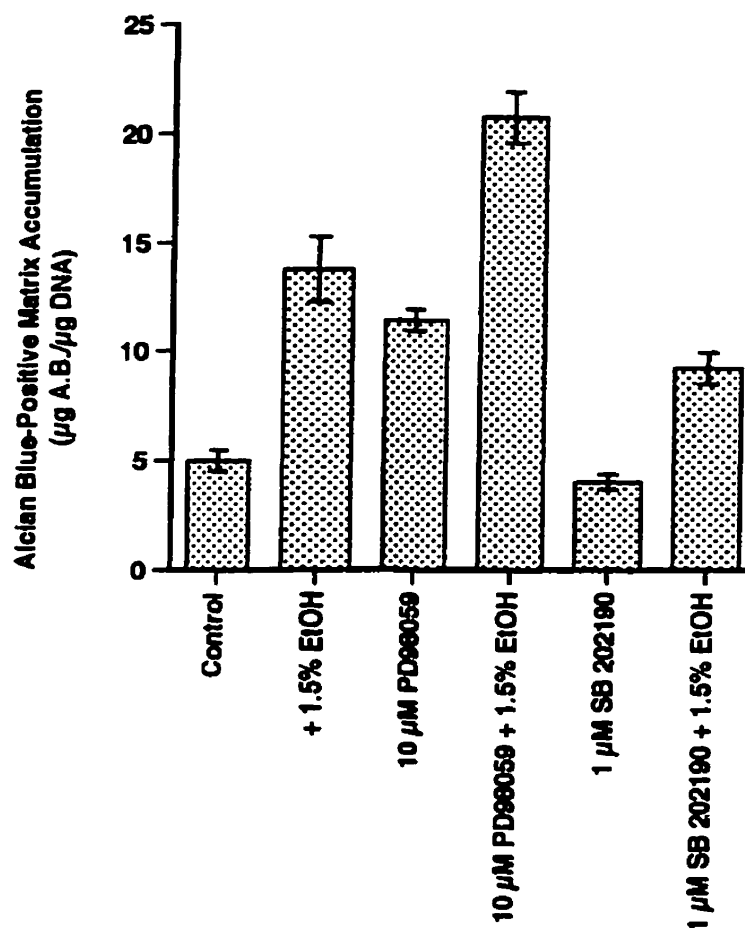


Figure 34 Graph quantifying the influence of various MAP Kinase inhibitors on ability of ethanol to enhance sulfated GAG accumulation in micromass cultures of limb mesenchyme.

Levels of Alcian blue-positive matrix accumulation/ μg DNA were examined in micromass cultures that were prepared from stage 23/24 whole wing bud mesenchyme and incubated for 3 days in control medium; 3 days in medium containing 10 μM PD98059 alone or 1 μM SB202190 alone; a 12 hour preincubation in control medium, followed by 48 hours in 1.5% ethanol; or a 12 hour preincubation with each of the inhibitors alone, followed by 48 hours in the presence of both inhibitor and 1.5% ethanol. Bars represent the mean \pm standard deviation of measurements from four replicate cultures.

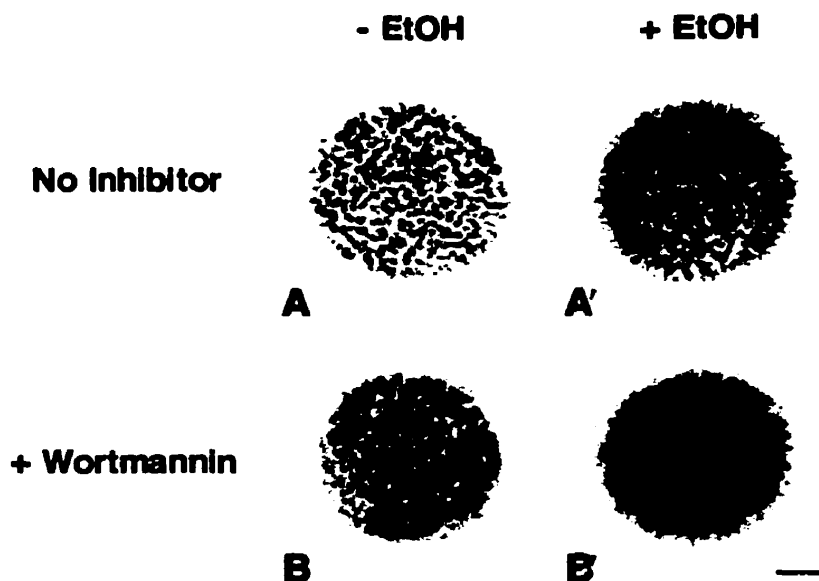


Figure 35 Photomicrographs demonstrating the effect of Wortmannin on ability of ethanol to enhance Alcian blue-positive matrix accumulation *in vitro*.

Micromass cultures of mesenchyme cells were isolated from whole stage 23/24 wing buds and plated at 2×10^5 cells/ $10\mu\text{l}$ medium. Cultures were then stained with Alcian blue, pH 1, following 3 days of incubation in control medium (A); 3 days in medium containing $1\mu\text{M}$ Wortmannin alone (B); a 12 hour preincubation in control medium followed by 48 hours in 1.5% ethanol (A'); or a 12 hour preincubation with $1\mu\text{M}$ Wortmannin, followed by 48 hours in the presence of both inhibitor and 1.5% ethanol (B'). Scale bar, 1 mm.

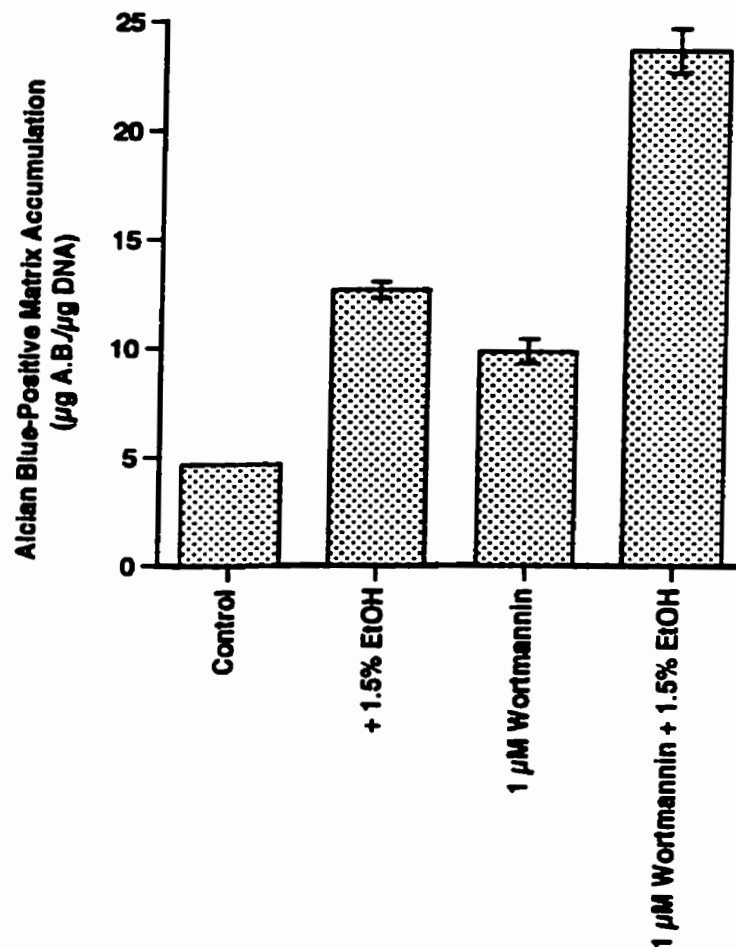


Figure 36 Graph illustrating the effect of Wortmannin on ability of ethanol to enhance sulfated GAG accumulation in micromass cultures of limb mesenchyme.

Levels of Alcian blue-positive matrix accumulation/ μg DNA were examined in micromass cultures prepared from stage 23/24 whole wing bud mesenchyme and incubated for 3 days in control medium; 3 days in medium containing 1 μM Wortmannin alone; a 12 hour preincubation in control medium, followed by 48 hours in 1.5% ethanol; or a 12 hour preincubation with 1 μM Wortmannin, followed by 48 hours in the presence of both inhibitor and 1.5% ethanol. Bars represent the mean \pm standard deviation of measurements from four replicate cultures.

with both Wortmannin and ethanol elaborated conspicuously greater Alcian blue-positive cartilage matrix (Figure 35B' and Figure 36) than those treated with Wortmannin alone (significant at $P < 0.001$). In contrast, 1 μM SB202190, a potent inhibitor of p38 MAP kinase (but not of ERK) (Jiang et al., 1997; Manthey et al., 1998), did not exert any stimulatory effect on the differentiation of limb mesenchyme cells. Rather, there was a suppression of total Alcian blue-positive matrix accumulation (Figure 33C). This effect appeared to be a result of SB202190's cytotoxicity on limb mesenchyme cells since quantitative analysis of Alcian blue-positive matrix accumulation did not reveal a significant effect of this agent on *in vitro* cartilage formation relative to untreated control cultures when normalized against cellular DNA content (Figure 34)($P > 0.05$). When cultures were co-treated with SB202190 and ethanol, chondrogenesis was markedly enhanced (Figure 33C' and Figure 34). Similar results were obtained in a replicate experiment. Together, these results suggest that ethanol may not require active ERK or p38 MAP kinase pathways, nor an active PI-3 kinase signaling pathway, to stimulate cartilage differentiation *in vitro*.

3.5.4 Protein Kinase A (PKA) Inhibitors

Micromass cultures of prechondrogenic limb mesenchyme incubated in the presence of the PKA inhibitors, 1 μM H-89 (Figure 37B and Figure 38) and 0.5 μM myristoylated PKA Inhibitor amide (N-Myr-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NH₂) demonstrated levels of Alcian blue-positive cartilage matrix accumulation similar to parallel control cultures (Figure 37A,C and Figure 38) ($P > 0.05$; ANOVA, Tukey's multiple comparisons test). Both agents have been shown to be potent inhibitors of PKA ($K_i = 48 \text{ nM}$ and 1.7 nM , respectively), although H-89 may also inhibit other ser/thr kinases at concentrations greater than 30 μM (Cheng et al., 1986; Glass et al., 1989; Chijiwa et al., 1990; Geilen et al., 1992). Cultures that were cotreated with 1.5% ethanol and either H-89 or PKA Inhibitor amide produced a significantly greater amount of Alcian blue cartilage matrix (Figure 37B',C' and Figure 38) ($P < 0.001$), similar to parallel cultures incubated with ethanol alone (Figure 37A' and Figure 38). PKA Inhibitor amide was tested once, whereas H-89 was examined in three independent experiments. These results suggest that ethanol does not require the PKA pathway to stimulate cartilage differentiation in micromass culture.

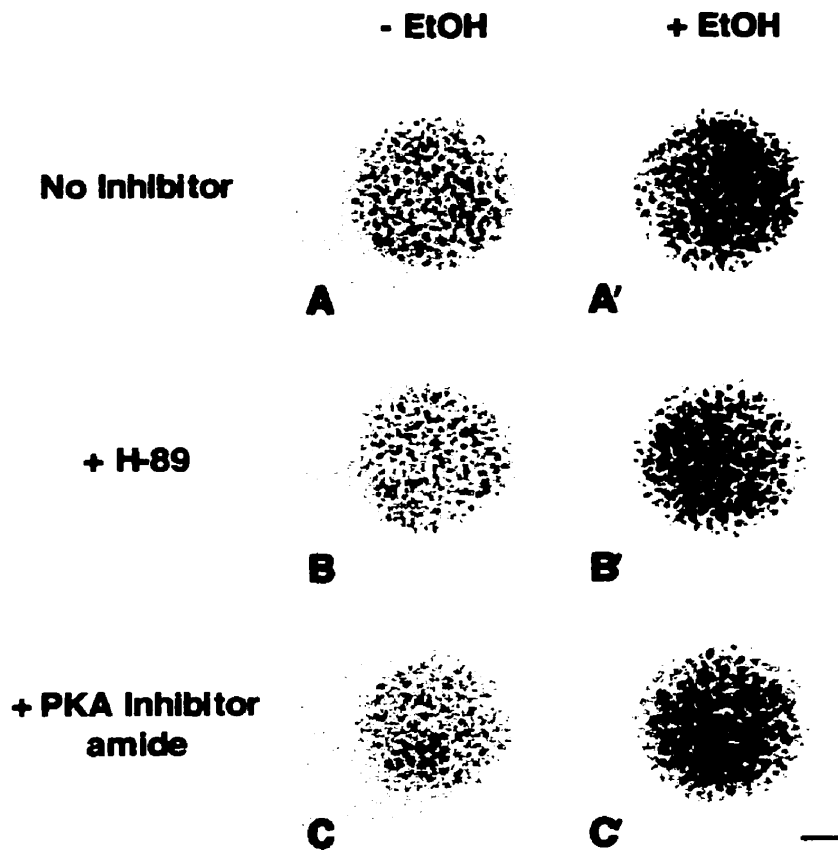


Figure 37 Photomicrographs illustrating effects of various Protein Kinase A (PKA) inhibitors on ability of ethanol to enhance Alcian blue-positive cartilage matrix accumulation *in vitro*.

Micromass cultures of mesenchyme cells from whole stage 23/24 wing buds were plated at 1×10^5 cells/ $10\mu\text{l}$ medium. Cultures were subsequently stained with Alcian blue, pH 1, following 3 days of incubation in control medium (A); 3 days in medium containing $1\mu\text{M}$ H-89 alone (B) or $0.5\mu\text{M}$ PKA inhibitor amide alone (C); a 12 hour preincubation in control medium followed by 48 hours in 1.5% ethanol (A'); or a 12 hour preincubation with each of the inhibitors alone, followed by 48 hours in the presence of both inhibitor and 1.5% ethanol (B', C'). Scale bar, 1 mm.

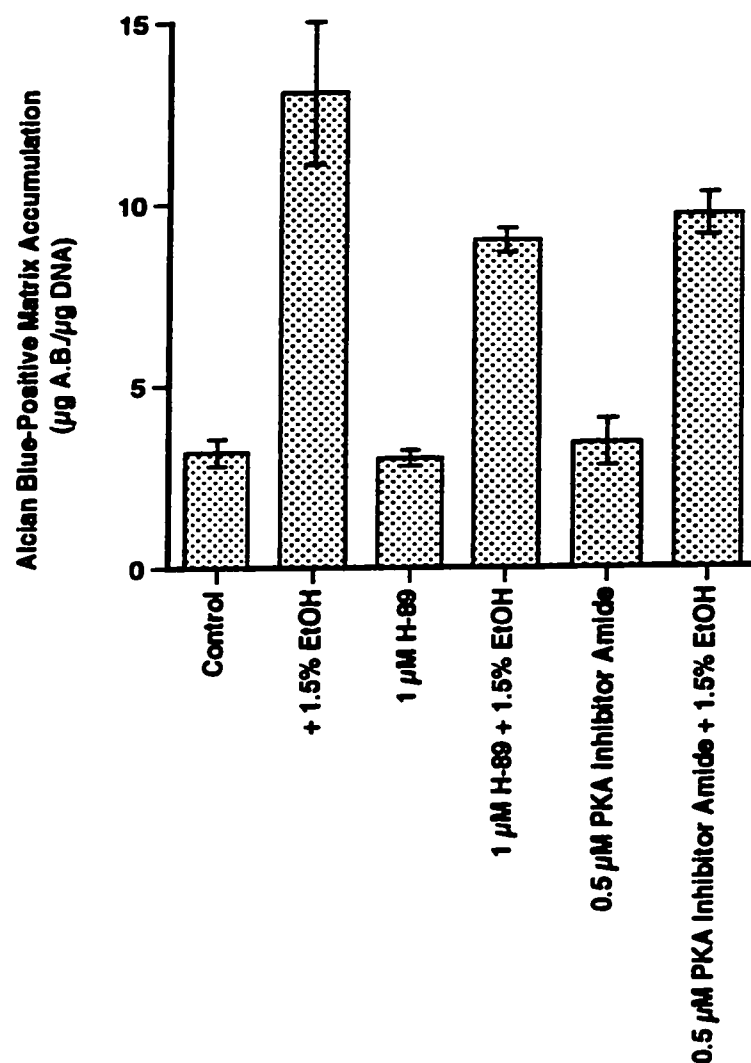


Figure 38 Graph quantifying effects of various PKA inhibitors on ability of ethanol to enhance sulfated GAG accumulation in micromass cultures of limb bud mesenchyme.

Levels of Alcian blue-positive matrix accumulation/ μg DNA were examined in micromass cultures prepared from stage 23/24 whole wing bud mesenchyme and incubated for 3 days in control medium; 3 days in medium containing $1\ \mu\text{M}$ H-89 alone or $0.5\ \mu\text{M}$ PKA inhibitor amide alone; a 12 hour preincubation in control medium, followed by 48 hours in 1.5% ethanol; or a 12 hour preincubation with each of the inhibitors alone, followed by 48 hours in the presence of both inhibitor and 1.5% ethanol. Bars represent the mean \pm standard deviation of measurements from four replicate cultures.

3.5.5 Protein Tyrosine Kinase (PTK) Inhibitors

I also tested several protein tyrosine kinase (PTK) inhibitors, 1 μ M Lavendustin A, 2 μ M Erbstatin, 5 μ M Tyrphostin 47, and 5 μ M Genistein, all of which have been shown to inhibit signaling through the EGF receptor and certain other tyrosine kinases (IC_{50} = 11 nM, 750 nM, 2.4 μ M and 2.6 μ M, respectively)(Akiyama et al., 1987; Yaish et al., 1988; Dvir et al., 1991; Hsu et al., 1991; Levitzki and Gazit, 1995; Clark et al., 1996; Koch et al., 1996). At concentrations below 100 μ M, these inhibitors are reported to have little or no effect on ser/thr kinases, including PKC and PKA. Lavendustin, Erbstatin, and Tyrphostin alone had no significant effect on cartilage matrix accumulation/ μ g DNA (Figure 39B,C, D and E, respectively and Figure 40) ($P > 0.05$). The effect of Genistein alone was variable: in two experiments, Genistein appeared to enhance cartilage matrix accumulation/ μ g DNA (e.g., Figure 40), while in another it had no significant effect relative to untreated control cultures. Regardless, when cultures were cotreated with 1.5% ethanol and either Lavendustin A, Erbstatin, Tyrphostin, or Genistein, cartilage differentiation was quantitatively enhanced (Figure 39B',C', D', E' and Figure 40) (significant at $P < 0.001$). Thus, none of the tyrosine kinase inhibitors tested blocked the ability of ethanol to elevate *in vitro* chondrogenesis. Experiments with Lavendustin A, Erbstatin and Tyrphostin were each performed once, whereas the effects of Genistein were examined in three independent experiments.

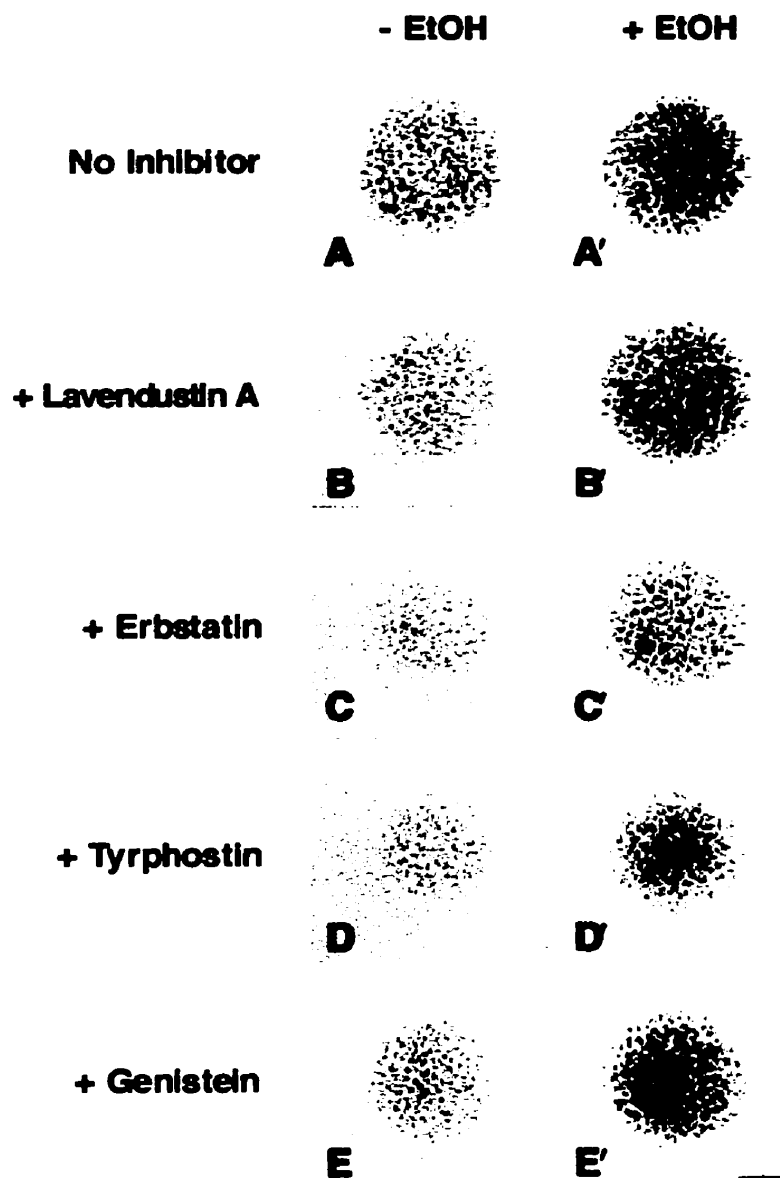


Figure 39 Photomicrographs demonstrating the influence of various Protein Tyrosine Kinase (PTK) inhibitors on ability of ethanol to enhance Alcian blue-positive cartilage matrix accumulation *in vitro*.

Micromass cultures of mesenchyme cells from whole stage 23/24 wing bud were plated at 1×10^5 cells/10 μ l medium. Cultures were then stained with Alcian blue, pH 1, following 3 days of incubation in control medium (A); 3 days in medium containing 1 μ M Lavendustin A alone (B), 2 μ M Erbstatin alone (C), 5 μ M Tyrphostin alone (D), or 5 μ M Genistein alone (E), a 12 hour preincubation in control medium followed by 48 hours in 1.5% ethanol (A'), or a 12 hour preincubation with each of the inhibitors alone, followed by 48 hours in the presence of both inhibitor and 1.5% ethanol (B', C', D', E'). Scale bar, 1 mm.

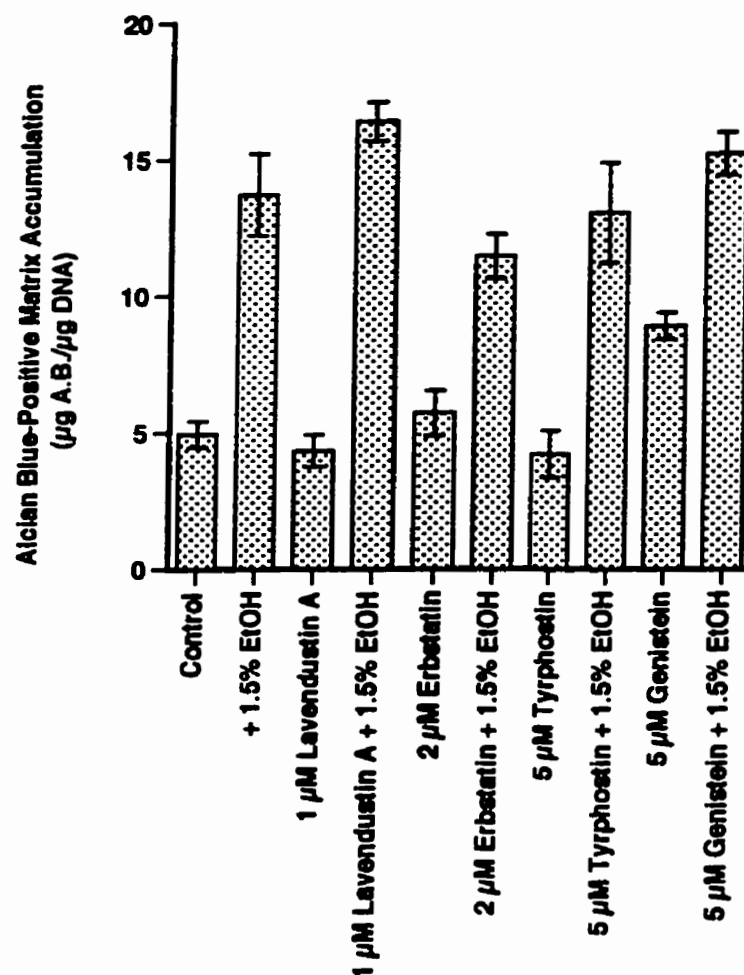


Figure 40 Graph illustrating the influence of various PTK inhibitors on ability of ethanol to enhance sulfated GAG accumulation in micromass cultures of limb bud mesenchyme.

Levels of Alcian blue-positive matrix accumulation/ μg DNA were examined in micromass cultures prepared from stage 23/24 whole wing bud mesenchyme and incubated for 3 days in control medium; 3 days in medium containing 1 μM Lavendustin A alone, 2 μM Erbetatin alone, 5 μM Tyrphostin alone or 5 μM Genistein alone; a 12 hour preincubation in control medium, followed by 48 hours in 1.5% ethanol; or a 12 hour preincubation with each of the inhibitors alone, followed by 48 hours in the presence of both inhibitor and 1.5% ethanol. Bars represent the mean \pm standard deviation of measurements from four replicate cultures.

4. DISCUSSION

It is well-recognized that alcohol, when administered during embryonic development, induces a broad spectrum of stage- and dose-dependent congenital malformations which include craniofacial, limb, and vertebral column dysmorphologies (Herrmann et al., 1980; Streissguth et al., 1980; Sulik et al., 1981; Webster et al., 1983; Pauli and Feldman, 1986; Webster and Ritchie, 1991). Despite an extensive literature documenting the deleterious effects of ethanol on vertebrate embryogenesis, the molecular mechanisms underlying its teratogenicity are still unclear. Many have attributed alcohol's pathogenicity to disturbances of systemic physiological processes (Streissguth et al., 1980; Sulik et al., 1981; Webster, 1989; Schenker et al., 1990; Kotch et al., 1992; Kotch and Sulik, 1992; Zajac and Abel, 1992). However, the relative extents to which the teratogenic effects of alcohol are due to systemic disturbances of embryonic growth and metabolism versus selective local effects on specific embryonic progenitor cell populations, such as those initiating skeletogenesis, are currently unclear. Therefore, my thesis work has focused on examining the effects of alcohol exposure on chondrocyte differentiation in cultures of prechondrogenic mesenchyme cells isolated from the developing limb buds and facial primordia of the chicken embryo, and investigating the molecular pathways that may mediate ethanol's effects on *in vitro* chondrogenesis.

4.1 Ethanol Exposure Elevates *In Vitro* Chondrogenic Differentiation by Embryonic Limb Mesenchyme

The present study demonstrates that in the presence of 0.5 - 2.0% ethanol (v/v), the deposition of sulfated matrix proteoglycans is significantly increased in spontaneously-differentiating micromass cultures of prechondrogenic limb mesenchyme. Stimulation of cartilage matrix accumulation in limb mesenchyme cultures, as measured by $^{35}\text{SO}_4$ uptake per μg DNA into CPC-precipitable glycosaminoglycans (GAGs), was maximal at concentrations of 1.5 - 2% ethanol. Sulfated GAGs (chondroitin sulfate and keratan sulfate) are covalently linked to the core protein of aggrecan, a predominant component of cartilage extracellular matrix, and as such, this method provided a means to accurately quantify the effects of ethanol treatment on cartilage matrix accumulation with a high degree of sensitivity.

Histochemical staining with Alcian blue dye confirmed the stimulatory influence of ethanol treatment on cartilage differentiation since the total amount of Alcian blue-positive cartilage matrix was markedly enhanced in cultures exposed to 1.5 - 2.0% ethanol. Ethanol treatment also promoted an increase in the amount of Alcian blue dye bound to sulfated GAGs/ μ g DNA, corroborating my $^{35}\text{SO}_4$ -uptake data.

Further evidence that ethanol exposure stimulates chondrogenic differentiation by embryonic limb mesenchyme has come from the demonstration that steady-state levels of gene transcripts for type II collagen, the principal collagenous protein of cartilage matrix, were dramatically elevated in response to ethanol exposure. Aggrecan RNA levels were also increased following ethanol treatment, and paralleled the observed rise in sulfated GAG accumulation. Therefore, both aggrecan gene transcript levels and the synthesis of the aggrecan proteoglycan product are influenced by ethanol. Importantly, ethanol treatment rapidly enhanced the expression of transcripts for the *Sox-9* transcription factor that has been shown to directly activate type II collagen gene expression in chondrogenic tissues (Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997). Recent evidence suggests that *Sox-9* may also regulate the transcription of other chondrocyte-specific genes (Bridgewater et al., 1998; Xie et al., 1999). As such, *Sox-9* is believed to act as a "master switch" gene for chondrogenic differentiation and cartilage-characteristic gene activation.

The stimulatory effects of ethanol on chondrogenic differentiation by prechondrogenic limb mesenchyme *in vitro* were even more dramatic when the cells were plated at a subconfluent density (0.5×10^7 cells/ml), conditions in which the cells undergo relatively little cartilage differentiation spontaneously (Umansky, 1966; Ahrens et al., 1977; Kulyk et al., 1989b). Again, several lines of evidence support this finding. Ethanol promoted a significant increase in total deposition of Alcian blue-positive cartilage matrix, and in $^{35}\text{SO}_4$ -labeled GAG accumulation/ μ g DNA in low-density cultures of both whole limb mesenchyme and distal subridge mesenchyme. Moreover, in both whole limb mesenchyme and distal subridge mesenchyme cultures, levels of type II collagen, aggrecan, and *Sox-9* mRNAs were elevated in response to ethanol treatment. Cumulatively, these results provide strong evidence that ethanol does indeed stimulate cartilage differentiation of embryonic limb mesenchyme.

4.2 Stimulatory Effects of Ethanol are Not Restricted to Prechondrogenic Limb Mesenchyme

I have demonstrated that ethanol exerts similar stimulatory effects on chondrogenic differentiation in cultures of mesenchyme isolated from various facial primordia of the chicken embryo (maxillary, mandibular, frontonasal and hyoid processes). In all four populations of facial mesenchyme, the presence of ethanol (1% ethanol in mandibular and frontonasal mesenchyme cultures and 1.5% ethanol in maxillary and hyoid arch mesenchyme cultures) significantly enhanced the incorporation of sulfated GAGs into matrix proteoglycans. Total Alcian blue-positive cartilage matrix accumulation was also elevated. Moreover, in each population of facial mesenchyme, ethanol exposure enhanced expression of type II collagen and aggrecan mRNAs. These results indicate that alcohol acts at a pretranslational level in elevating cartilage matrix production by facial mesenchyme cells, as it does in the limb mesenchyme cultures.

It should be noted that whereas the chondrogenic mesenchyme cells of the limb buds are derived from somatic mesoderm, the entire facial skeleton differentiates from neural crest ectoderm (LeLievre, 1978; Noden, 1983; Couly et al., 1993). Thus, ethanol's stimulatory influence on chondrogenesis may be a property common to all embryonic cartilage progenitor cells, regardless of their specific ontogenetic lineage. In previous studies conducted in our laboratory, limb and facial mesenchyme were found to also respond similarly to staurosporine, a protein kinase inhibitor that enhances chondrogenic differentiation *in vitro* (Kulyk, 1991; Kulyk and Reichert, 1992). It is likely that there are many commonalities in the molecular pathways regulating morphogenesis and differentiation in the limb and facial skeletons, despite the differing origins of their skeletogenic progenitor cells (Helms et al., 1997).

4.3 Ethanol Directly Influences the Differentiation of Chondrogenic Progenitor Cells

The present study demonstrates that ethanol stimulates chondrogenesis even when cultures are prepared from distal tip, or subridge, mesenchyme of the stage 24/25 chicken embryo wing bud. Cells isolated from this region represent a more homogeneous population of prechondrogenic mesenchyme since myoblast progenitors are restricted to more proximal regions at this stage (Newman et al., 1981; Gay and Kosher, 1984). The stimulation was evident at both the level of cartilage matrix synthesis (sulfated GAG accumulation) and expression of cartilage-specific RNAs (type II collagen, aggrecan, and *Sox-9*). Thus, alcohol exposure appears to directly influence

the differentiation of the chondroprogenitor cells of the limb. Although a similarly pure population of prechondrogenic mesenchyme cells could not be isolated from the facial primordia of the chicken embryo, it is reasonable to assume that the neural crest cells are those responsive to ethanol's cartilage-inducing effects since they form all cartilage, bone, and connective tissues of the face *in vivo*. The remaining mesenchymal cells of the facial primordia are derived from paraxial mesoderm and appear to be committed to myogenic differentiation (Noden, 1983).

4.4 Ethanol's Stimulatory Effects on Chondrogenic Differentiation are a Result of Direct Action of the Alcohol Itself Rather than a Downstream Metabolite

My findings demonstrate that other alcohols (4% methanol and 1% propanol) were also able to enhance cartilage differentiation by embryonic limb mesenchyme. Moreover, exposure to 1% tertiary butanol, an alcohol which is not metabolized to an aldehyde (Williams, 1959; Wood and Laverty, 1979), was as effective as 2% ethanol in stimulating *in vitro* chondrogenesis. Like ethanol, the effects of tertiary butanol on chondrogenic differentiation were seen both at the level of cartilage matrix GAG accumulation and the level of expression of transcripts for type II collagen and aggrecan. The stimulatory effects of tertiary butanol were not only observed in limb mesenchyme cultures, but also in facial mesenchyme cultures. These results suggest that ethanol's stimulatory influence on cartilage differentiation is directly exerted by the alcohol itself, rather than its acetaldehyde metabolite. Several *in vivo* studies have similarly concluded that the teratogenic effects of ethanol on vertebrate embryogenesis are due to the alcohol itself (Schenker et al., 1990). For example, administration of alcohol in conjunction with inhibitors of ALDH, which should result in prolonged elevation of acetaldehyde levels, does not result in a significant increase in teratogenicity over the alcohol alone (Webster, 1983). In contrast, administration of both ethanol and the ADH inhibitor, pyrazole, results in an increased incidence of congenital malformations (Ukita et al., 1993).

4.5 Stimulatory Effects of Ethanol are Not Due to Altered Cellular Proliferation or Viability of Limb Mesenchyme Cells

At chondrogenically stimulatory concentrations of 0.5 - 1.5%, ethanol had little or no effect on total DNA accumulation (a measure of both cell viability and cell proliferation) in both spontaneously-differentiating, high-density cultures of limb mesenchyme and in weakly chondrogenic, low-density cultures of limb mesenchyme.

^3H -thymidine uptake (a measure of DNA synthetic activity) by limb mesenchyme cells was also not significantly altered by the presence of 1.5% ethanol in the culture medium. These results are consistent with a report by Cartwright et al. (1998) which demonstrated that *in vivo* ethanol exposure did not significantly alter cellular proliferation within cranial neural crest cell populations that contribute to the embryonic facial primordia. I have demonstrated, however, that concentrations exceeding 1.5% ethanol cause a sharp decline in the total DNA content of prechondrogenic limb mesenchyme cell cultures, suggesting that ethanol has cytotoxic effects at higher doses.

Cytosine arabinoside is an effective inhibitor of cellular proliferation in cultured limb mesenchyme cells, as previously reported by Biddulph et al. (1991), and verified by my own demonstration that it greatly reduces ^3H -thymidine uptake by limb mesenchyme cells. However, inhibition of cellular proliferation in cultured limb mesenchyme cells using cytosine arabinoside did not abolish the stimulatory effects of ethanol on chondrogenic differentiation. Rather, cultures that were co-treated with both cytosine arabinoside and ethanol exhibited conspicuously greater matrix accumulation than cultures treated with cytosine arabinoside alone. Thus, ethanol's stimulatory effects on chondrogenic differentiation are probably not dependent on active cellular proliferation.

4.6 Ethanol Enhances Chondrogenic Differentiation Only Under Conditions that Permit Some Degree of Spontaneous Chondrogenesis

Ethanol was able to stimulate chondrogenic differentiation by limb mesenchyme cells plated at a reduced cell density of 0.5×10^5 cells/10 μl drop of culture medium, conditions which still allow a low level of spontaneous cartilage formation. However, in preliminary experiments, I found that it was unable to exert a significant stimulatory effect on cartilage formation when cells were maintained under even lower-density culture conditions (0.25×10^5 cells/10 μl drop of medium) in which the cells did not undergo any spontaneous cartilage differentiation (data not shown). Ethanol's inability to significantly enhance chondrogenesis under these very low-density conditions is qualitatively similar to the effects of dibutyryl cAMP on chondrogenesis in low-density cultures of limb mesenchyme. This cAMP analogue was shown to stimulate cartilage differentiation only when limb mesenchyme cells were cultured at high cell densities that allowed close cellular juxtapositions and gap junctional communication (Rodgers et al., 1989; Coelho and Kosher, 1991). The possibility that ethanol may also act to modulate the cell-cell interactions that activate cartilage-specific gene expression remains to be addressed. However, the findings of the present study suggest that a

certain threshold level of spontaneous chondrogenesis may be required for ethanol to exert its effects on cartilage differentiation.

Ethanol was also unable to significantly enhance the deposition of Alcian blue-stainable extracellular matrix and sulfated GAG accumulation/ μ g DNA in high-density micromass cultures of prechondrogenic limb mesenchyme that had been incubated in the presence of low (1%) FBS, conditions that are not favorable for spontaneous cartilage formation. Serum is believed to contain a variety of polypeptide growth factors, hormones, and other soluble molecules (e.g., IGFs, PDGFs, FGFs, thyroid hormones, steroid hormones, vitamin D, prostaglandins, retinols, etc.; Freshney, 1987; HyClone Laboratories, personal communication) that have been implicated as regulators of chondrogenesis (reviewed in Cancedda et al., 1995). Accordingly, it seems reasonable to suggest that ethanol may stimulate cartilage differentiation *in vitro* by modulating the response of prechondrogenic mesenchyme cells to one or more specific serum factors.

Ethanol also promoted only a slight increase in chondrogenic differentiation in micromass cultures of limb mesenchyme that were pre-incubated with PMA, a phorbol ester that strongly suppresses cartilage differentiation by embryonic limb mesenchyme cells under high-density culture conditions that are otherwise permissive for cartilage formation (Sasse et al., 1983; Kulyk, 1991). Matrix formation and levels of expression of type II collagen mRNA did not recover to the level observed in untreated control cultures, and certainly not to the level observed in ethanol-treated cultures of prechondrogenic limb mesenchyme. Thus, ethanol is not able to substantially overcome the suppressive effects of PMA on chondrogenesis, providing still further evidence that alcohol may exert potent stimulatory effects on chondrogenic differentiation only under conditions that permit some degree of spontaneous chondrogenesis.

Cumulatively, the results of the present study suggest that ethanol may enhance or accelerate, rather than induce, chondrogenic differentiation in cultures of prechondrogenic limb mesenchyme. Indeed, as will be discussed below, ethanol treatment appears to accelerate the changes in gene expression (e.g., type II collagen, aggrecan, and *Sox-9*) that normally occur during chondrogenesis.

Ethanol was, however, able to stimulate cartilage matrix accumulation, and elevate the expression of transcripts for type II collagen and aggrecan in maxillary and hyoid arch mesenchyme cell cultures which form little or no cartilage spontaneously (Wedden et al., 1986; Kulyk and Reichert, 1992). As such, it is not as clear whether alcohol also requires some degree of spontaneous chondrogenesis to exert its stimulatory effects on chondrogenic differentiation in cultures of neural crest-derived

facial mesenchyme. Despite their markedly different tendencies for spontaneous chondrogenic differentiation when cultured *in vitro*, each of the four embryonic facial primordia examined in the present study has at least some capacity to form cartilage *in vivo*, although the relative extents of osteogenic versus chondrogenic differentiation in these facial primordia differ considerably. For example, in vertebrate embryos, the prenasal cartilage of the upper jaw or beak, Meckel's cartilage of the mandible, and the cartilaginous template of the hyoid bone are formed by endochondral ossification within the frontonasal, mandibular, and hyoid processes, respectively. In contrast, the mandible *per se* is formed by intramembranous ossification of the mesenchymal tissue surrounding Meckel's cartilage. Most derivatives of the maxillary process are similarly formed by intramembranous ossification, although secondary cartilages can form at sites of apposition between membrane bones (Noden, 1983; Couly et al., 1993). The ability of ethanol to induce cartilage nodule formation in maxillary and hyoid arch mesenchyme cultures may reflect the latent chondrogenic potential of skeletogenic mesenchyme cells in these primordia.

4.7 Ethanol Appears to Act at an Early Point in the Chondrocyte Differentiation Pathway

The stimulatory effects of alcohol on *in vitro* cartilage differentiation appear to be restricted to *prechondrogenic* mesenchyme cells. When differentiated chondrocytes isolated from either the cranial or caudal portions of 16 - 18 day old chick embryo sterna were exposed to 1.0 - 1.5% ethanol, neither Alcian blue-positive matrix accumulation nor the level of expression of type II collagen and aggrecan mRNAs were elevated. Instead, ethanol treatment exerted either little effect, or modestly suppressed chondrogenic differentiation in sternal chondrocyte cultures. These results suggest that alcohol does not act by enhancing levels of cartilage matrix synthesis and cartilage-specific gene expression in cells that have already undergone overt, phenotypic differentiation into chondrocytes. Rather, ethanol selectively promote the *initial* differentiation of prechondrogenic mesenchyme cells into chondrocytes. My observation that ethanol treatment modestly suppressed type II collagen and aggrecan transcript levels in cultures of sternal chondrocytes led me to further question whether ethanol might accelerate chondrocyte hypertrophy and endochondral ossification, since a decline in type II collagen and aggrecan expression accompanies this process (Oshima et al., 1989). However, ethanol treatment also suppressed the expression of the type X collagen gene in cultures of cranial sternal chondrocytes. Since the appearance of type X collagen mRNA and protein is a definitive marker of the shift to hypertrophy (Gibson

and Flint, 1985; Oshima et al., 1989), it seems unlikely that ethanol accelerates the progression of sternal chondrocytes toward this stage of terminal differentiation. Accordingly, the results of my study suggest that alcohol may only be capable of exerting stimulatory effects on cartilage matrix synthesis and gene expression in mesenchymal *chondroprogenitor* cells.

Levels of type II collagen and aggrecan mRNAs were quantitatively elevated in prechondrogenic limb mesenchyme cells within 24 hours of exposure to ethanol. This observation lends further support to our conclusion that ethanol may act at an early point in the chondrocyte differentiation pathway. Moreover, ethanol treatment rapidly altered the expression of transcripts for several putative chondrogenic regulatory genes (*Sox-9*, *BMPR-IB*, *Ets-2*, and *TGF- β_3*) that have been implicated in the initial mesenchyme cell condensation phase of chondrogenesis and/or the activation of genes for the structural components of cartilage matrix.

Evidence suggests that the *Sox-9* transcription factor plays a particularly crucial role in chondrogenesis. Indeed, it has been shown to be co-expressed with type II collagen in prechondrogenic mesenchyme both *in vivo* and *in vitro* (Wright et al., 1995; Ng et al., 1997; Zhao et al., 1997; Healy et al., 1999; Kulyk et al., 2000). The appearance of *Sox-9* gene transcripts temporally precedes the upregulation of type II collagen gene expression in mesenchyme cell aggregates (Ng et al., 1997; Zhao et al., 1997; Kulyk et al., 2000). Moreover, a chondrocyte-specific enhancer in the type II collagen gene, *Col2A1*, is a direct target of the *Sox-9* protein (Bell et al., 1997; Lefebvre et al., 1997). Thus, it appears that *Sox-9* acts upstream of the type II collagen gene in the chondrocyte differentiation pathway. The present study demonstrates that in limb mesenchyme cultures maintained at both high- and low-density, ethanol treatment rapidly upregulated mRNA expression of the *Sox-9* transcription factor. The stimulatory effect of ethanol on *Sox-9* gene expression preceded the effect of ethanol on type II collagen gene expression. Indeed, as early as 3 - 8 hours following ethanol exposure, micromass cultures of distal limb mesenchyme demonstrated quantitatively greater levels of *Sox-9* mRNAs than parallel control cultures. These results suggest that ethanol acts upstream of *Col2A1* which is normally upregulated coincident with the onset of condensation and overt chondrogenesis. Whether ethanol acts directly upon *Sox-9* gene expression, or influences genes acting upstream of *Sox-9* in the chondrocyte differentiation pathway remains to be elucidated.

Recent evidence suggests that *Sox-9* may be under the control of a Bone Morphogenetic Protein (BMP)-dependent signaling pathway, since ectopic expression of noggin, a BMP antagonist, results in the loss of *Sox-9* gene expression in developing

limbs *in vivo* (Healy et al., 1999). Further evidence that BMP signaling may be important in chondrogenic differentiation has come from the demonstration that the BMP receptor, BMPR-IB, is expressed in prechondrogenic cell aggregates prior to overt cartilage differentiation (Kawakami et al., 1996; Zou et al., 1997). Moreover, expression of dominant-negative BMPR-IB has demonstrated that BMPR-IB activity is essential for cartilage formation to occur both *in vivo* and *in vitro* (Kawakami et al., 1996; Zou et al., 1997). I have demonstrated that mRNA levels of BMPR-IB are upregulated in micromass cultures of limb mesenchyme following exposure to 1.5% ethanol. This upregulation of gene expression did not occur as rapidly as for *Sox-9*. Levels of BMPR-IB mRNAs were quantitatively elevated following 16 - 24 hours incubation in ethanol-treated medium, immediately preceding the upregulation of *Col2A1* expression that was observed 24 - 40 hours after ethanol treatment. This is consistent with the demonstration by Zou et al. (1997) that BMPR-IB expression temporally precedes that of type II collagen in the developing chick limb bud *in vivo*. More importantly, my data provide further evidence to support our conclusion that ethanol exerts its effects at an early point in the chondrocyte differentiation pathway.

Growth factors of the TGF- β family have also been implicated as potential regulators of chondrogenic differentiation. When added exogenously to cultured cells, members of the TGF- β family induce or enhance cartilage matrix synthesis. For example, exogenous TGF- β_1 promotes cartilage differentiation by fibroblastic cells isolated from fetal rat muscle explants (Seyedin, 1985; Seyedin, 1986; Seyedin, 1987), and chick limb mesenchyme cells plated at subconfluent cell densities that do not favor spontaneous chondrogenesis (Kulyk et al., 1989b). However, ethanol treatment rapidly downregulated endogenous TGF- β_3 expression in our limb mesenchyme cell cultures, and had no discernible effect on TGF- β_2 and TGF- β_4 mRNAs. We were initially surprised by these results, having expected that ethanol might stimulate chondrogenesis by elevating the expression of TGF- β gene transcripts in micromass culture. However, a study by Roark and Greer (1994) has demonstrated that the levels of expression of all three TGF- β mRNAs are highest prior to or during the initial prechondrogenic aggregation phase of chondrogenesis in micromass cultures, and then decline when cartilage matrix molecules, type II collagen and aggrecan, begin to accumulate. Since ethanol treatment promotes the precocious expression of type II collagen and aggrecan genes, it seems plausible to suggest that ethanol might correspondingly accelerate the downregulation of TGF- β_3 RNA expression that normally occurs during *in vitro* chondrogenesis.

Members of the *Ets* superfamily of transcription factors have also been found to be expressed in cartilaginous primordia *in vivo* (Maroulakou et al., 1994; Dhordain et al., 1995). The potential importance of *Ets-2*, in particular, in regulating cartilage formation is substantiated by reports that overexpression of *Ets-2* in transgenic mice results in craniofacial skeletal abnormalities resembling those of Down's Syndrome (Sumarsono et al., 1996). In the present study, *Ets-2* gene transcript expression was found to be elevated in response to ethanol exposure. This upregulation occurred coincident with the upregulation of *BMPR-IB* gene expression. In contrast, ethanol treatment had no significant effect on the expression of gene transcripts for *Erg*, another member of the *Ets* superfamily.

4.8 Ethanol Treatment Does Not Alter *Msx-2* Gene Expression in Cultures of Prechondrogenic Limb Mesenchyme

Evidence suggests that *Msx-2* may be involved in regulating AER activity, in delineating the nonchondrogenic regions of the developing limb, and in programmed cell death (e.g., in the interdigital regions) (Coelho et al., 1991; Ferrari et al., 1998). It is not expressed in progress zone mesoderm, the region of highly proliferative mesodermal cells underneath the AER which will subsequently undergo chondrogenic differentiation and give rise to the skeletal elements of the limb (Coelho et al., 1991; Ferrari et al., 1998). Therefore, there appears to be an inverse relationship between *Msx-2* expression and chondrogenic differentiation.

Interestingly, ethanol treatment inhibits the expression of *Msx-2* RNA in cultured calvarial osteoblasts, and completely abolishes *Msx-2* expression in developing mouse embryos *in vivo* (Rifas et al., 1997). Thus, it has been postulated that inhibition of *Msx-2* expression may be a mechanism of action for FAS/FAE (Rifas et al., 1997). However, a study by Cartwright et al. (1998) has demonstrated that in a chick embryo model of FAS, ethanol treatment does not affect *Msx-2* gene expression in cranial neural crest cells. The results of my own study are consistent with this finding. I have demonstrated that *Msx-2* gene expression is unaffected in cultured chick limb mesenchyme cells following exposure to ethanol. Although this could reflect differences in the ethanol-responsiveness of tissues from different species (e.g., bird versus mammal), we were initially surprised by these results, having expected that ethanol might stimulate chondrogenesis by downregulating the expression of *Msx-2* gene transcripts in micromass cultures (i.e., suppression of a cartilage suppressor). Clearly, further work must be done to elucidate the potential role of *Msx-2* in ethanol-

induced teratogenicity. My data suggests, however, that ethanol treatment does not stimulate *in vitro* chondrogenesis by altering the level of endogenous *Msx-2* expression.

4.9 Ethanol Treatment Does Not Elicit a Classical Stress Response

The expression of Hsp stress response genes may be rapidly activated and/or upregulated in cells following exposure to alcohol (Li, 1983; Hahn et al., 1985; Neuhaus-Steinmetz et al., 1993). I have demonstrated, however, that in cultured limb mesenchyme cells, ethanol treatment was unable to significantly activate the expression of a stress-inducible form of Hsp70, whereas a 45°C heat-shock rapidly induced Hsp70 expression. The fact that this particular form of Hsp70 is not constitutively expressed in untreated control cultures made it a particularly useful probe for investigating whether ethanol exerted its stimulatory effects on chondrogenesis via induction of a classic stress response. In contrast, both Hsp47 and Hsp90 α were found to be expressed at low levels in control cultures. This is not surprising since, under non-stress conditions, Hsp47 shows coordinately regulated patterns of expression with fibril-forming collagen types (Nagata, 1996; Lele and Krone, 1997), including type II collagen which is upregulated during chondrocyte differentiation. Unlike Hsp70, the level of expression of Hsp47 mRNA was modestly elevated following ethanol exposure. This is probably attributable to the fact that ethanol elevates levels of type II collagen expression in micromass cultures of limb mesenchyme. Similarly, ethanol treatment also promoted a modest increase in the level of expression of Hsp90 α RNA in micromass cultures of whole wing bud mesenchyme. These cultures contained myoblast progenitor cells in addition to chondrogenic progenitors. Since Hsp90 α is expressed in differentiating myogenic cells (Sass and Krone, 1997), it is possible that the slightly higher levels of Hsp90 α expression in ethanol-treated cultures may reflect an effect of ethanol on premyogenic cells rather than prechondrogenic cells. This possibility could be examined experimentally by utilizing distal subridge limb mesenchyme cultures which are devoid of myogenic precursor cells. Alternatively, one could directly examine the effects of ethanol treatment on expression of myogenic differentiation markers such as MyoD. Cumulatively, the results of the present study suggest that the ethanol concentrations that are effective in stimulating *in vitro* chondrogenesis do not elicit a significant stress response in prechondrogenic limb mesenchyme cells.

4.10 The Role of Specific Intracellular Signal Transduction Pathways in Mediating Ethanol's Effects on Chondrogenesis is Unclear

At present, the molecular mechanisms underlying alcohol's striking stimulatory effects on *in vitro* cartilage differentiation remain to be elucidated. However, studies on cultured neural, hepatic, and other cell types have demonstrated that ethanol exposure can modulate a variety of signal transduction pathways (Hoek and Rubin, 1990). Some of the diverse cellular effects of alcohols are associated with their ability to alter the fluidity of biological membranes (Littleton, 1989), and thereby influence an array of critical membrane-associated signaling processes (Taraschi and Rubin, 1985; Hoek and Rubin, 1990). In addition, ethanol may exert more direct and selective effects on some cell functions through actions at particular lipid-protein interfaces (Hoffman and Tabakoff, 1990), or through specific interactions with the hydrophobic domains of regulatory proteins such as protein kinase C (Slater et al., 1993). The alcohol-sensitive signaling pathways include many that have been implicated in the regulation of embryonic limb cartilage differentiation. For example, the activation of membrane receptor kinases for growth factors of the FGF, TGF- β , PDGF, IGF and BMP families is believed to regulate the formation of cartilage during limb morphogenesis *in vivo* (Centrella et al., 1988; Kulyk et al., 1989b; Leonard et al., 1991; Tabin, 1991; Niswander and Martin, 1993a; Savage et al., 1993; Laufer et al., 1994; Dealy and Kosher, 1995), and probably also mediates the differentiative responses of cultured limb mesenchyme cells to serum growth factors. Elevated prostaglandin production (Chepenik et al., 1984; Biddulph et al., 1988; Capehart and Biddulph, 1991), and an ensuing rise in intracellular cyclic AMP (Elmer et al., 1981; Ho et al., 1982; Kosher and Walker, 1983) are associated with prechondrogenic mesenchyme cell condensation and chondrogenic differentiation in the developing limb. Modulations of protein kinase C activity (Garrison et al., 1987; Kulyk, 1991; Sonn and Solursh, 1993), and intracellular Ca^{2+} levels (Bouakka et al., 1988) in cultured limb mesenchyme cells have also been demonstrated to influence the extent of cartilage differentiation. Influences upon one or a combination of these processes might account for ethanol's stimulatory effects on cartilage differentiation *in vitro*.

It has been postulated that prenatal ethanol exposure may perturb embryonic morphogenesis *in vivo* by competing with retinol (vitamin A) for alcohol dehydrogenase (ADH) (Duester, 1991; Deltour et al., 1996). It is unlikely, however, that a competition for cellular ADH is responsible for the effects of ethanol on *in vitro* limb cartilage differentiation demonstrated in the present study. Tertiary butanol, which is neither a substrate for ADH nor metabolized to an aldehyde (Williams, 1959; Wood

and Lavery, 1979), was found to be as effective as ethanol at enhancing cartilage differentiation in limb and facial mesenchyme cultures. Together, these results suggest that ethanol's stimulatory influence on cartilage differentiation is directly exerted by the alcohol itself rather than its acetaldehyde metabolite. However, this doesn't discount the possibility that ethanol may interfere with retinoid signaling via some other mechanism.

As a first step toward examining the intracellular signaling mechanisms through which ethanol might exert its stimulatory effects on chondrogenesis, I employed a variety of pharmacological agents that inhibit specific intracellular kinase pathways. Cultures were then co-treated with ethanol to determine whether ethanol was able to enhance cartilage differentiation following the functional inactivation of a particular pathway.

4.10.1 Protein Kinase C (PKC) Inhibitors/Downregulators

Two different methods were used to block intracellular signaling through the PKC pathway. The first method employed agents that inhibit the kinase activity of the PKC enzyme, whereas the second method employed agents that cause a proteolytic depletion or "downregulation" of intracellular PKC. Since the PKC inhibitors and the PKC downregulators exerted considerably different effects on chondrogenic differentiation in cultures of prechondrogenic limb mesenchyme, it is difficult to interpret what role, if any, the PKC signaling pathway may play in either spontaneous chondrogenesis or in ethanol-induced chondrogenesis. For example, none of the PKC inhibitors tested (Bisindolylmaleimide, Chelerythrine, H-7) significantly affected chondrogenesis in micromass cultures of limb mesenchyme, relative to controls. Previous authors have reported that Bisindolylmaleimide, and two other PKC inhibitors, Go6976 and Calphostin C, suppress cartilage formation and the expression of type II collagen gene transcripts in cultures of limb mesenchyme (Martiny-Baron et al., 1993; Choi et al., 1995). However, a major methodological difference between the present study and the studies of Choi et al. (1995), in particular, was the length of time that limb mesenchyme cells were cultured in the presence of these agents. The data of Choi et al. indicate that Bisindolylmaleimide was unable to exert a significant effect on chondrogenic differentiation following a 3 day culture period, consistent with my own findings. Only after a more prolonged 6 day incubation in the presence of Bisindolylmaleimide did the limb mesenchyme cells demonstrate significantly less total cartilage matrix. Another major methodological difference between the report by Choi et al. (1995) and the present study is the concentration of Bisindolylmaleimide

employed. We selected a concentration of 0.5 μM Bisindolylmaleimide since it exceeded the dose reported to selectively inhibit PKC ($K_i = 10 \text{ nM}$), but was low enough to prevent nonspecific inhibition of other signaling pathways. However, the concentration (20 μM) utilized by Choi et al. (1995) has also been reported to inhibit PKA ($K_i = 2 \text{ } \mu\text{M}$). In addition, to compensate for the possibility that Bisindolylmaleimide might affect cell proliferation or viability, I normalized the Alcian blue-positive cartilage matrix accumulation of each sample against the average DNA content of parallel cultures. This was not done in the experiments by Choi et al. (1995) and as such, Bisindolylmaleimide may have merely caused a significant drop in total cell number, reflecting possible cytotoxic effects of this agent at high, physiologically-irrelevant doses.

More importantly, I have demonstrated that the presence of these PKC inhibitors did not inhibit ethanol's stimulation of chondrogenic differentiation *in vitro*. Rather, cultures of limb mesenchyme that were co-treated with ethanol and either Bisindolylmaleimide, Chelerythrine, or H-7 exhibited quantitative increases in the accumulation of sulfated GAGs into histochemically-identifiable cartilage matrix, relative to cultures that were treated with each of the inhibitors alone. The extent of this stimulation was similar to that seen between control and ethanol-treated cultures. Taken by themselves, the results from my PKC inhibitor studies seem to suggest that PKC is not required for ethanol to exert its stimulatory effects on this process.

However, the data obtained using PKC downregulators suggest a somewhat different interpretation. PMA, Mezerein, and OIL all dramatically suppressed cartilage differentiation by embryonic chick limb mesenchyme cells in micromass culture, confirming earlier reports by Jia (1995) and Choi et al. (1995). Each of these agents have been shown to cause a downregulation of endogenous PKC activity at the concentrations used in my study (Sonn and Solursh, 1993; Jia, 1995). Together, these studies suggest that PKC might be required for spontaneous chondrocyte differentiation *in vitro*. Interestingly, in cultures that were co-treated with both ethanol and either PMA, Mezerein or OIL, there was only a slight increase in chondrogenic differentiation, relative to cultures that were incubated in the presence of each of the PKC downregulators alone. Unlike the results of the PKC inhibitor studies, these results suggest the possibility that PKC may also play a role in mediating ethanol's stimulatory effects on cartilage differentiation *in vitro*. However, although PKC is considered to be the prime target of PMA and other phorbol esters (Castagna et al., 1982; Niedel et al., 1983; Edelman, 1987; Martelly and Castagna, 1989; Jaken, 1990; Roivainen and Messing, 1993), there is mounting evidence that it is not their exclusive

target. Indeed, it has been reported that secretion from rat lacrimal glands is stimulated by PMA, but not by analogs of the physiological PKC activator, diacylglycerol (Zoukhri et al., 1993). Moreover, the PMA-induced secretion is not inhibited by the selective PKC inhibitor, Chelerythrine, but is partially inhibited by staurosporine, a non-specific protein kinase inhibitor, and is completely inhibited by trifluoperazine, a calcium/calmodulin-dependent protein kinase inhibitor (Zoukhri et al., 1993). Thus, the possibility remains that PMA, Mezerein, and OIL may be suppressing cartilage differentiation by a mechanism independent of the PKC signal transduction pathway. In addition, the observation that ethanol treatment allowed only a partial recovery of chondrogenic differentiation may be attributable to the fact that, as discussed in a previous section, ethanol appears to enhance chondrogenesis only under conditions that favor spontaneous cartilage formation.

4.10.2 Mitogen-Activated Protein (MAP) Kinase Inhibitors

ERK MAP kinase subtypes are believed to play crucial regulatory roles in cytodifferentiation. Depending on specific isoforms and the cell type, these signaling pathways may either inhibit or stimulate differentiation. For example, activation of ERK MAP kinase has been shown to be required for the differentiation of fibroblasts into adipocytes (Sale et al., 1998). In contrast, inactivation of this signaling pathway is necessary for C2C12 myoblasts to initiate myogenesis (Bennett and Tonks, 1997). In micromass cultures of prechondrogenic limb mesenchyme, inhibition of Erk-1 MAP kinase with the pharmacological agent, PD98059, was reported to enhance chondrogenesis (Chang et al., 1998). The results of my own study are consistent with this finding. Thus, inactivation of the ERK MAP kinase signaling pathway may be required to initiate chondrocyte differentiation. In the present study, prechondrogenic limb mesenchyme cell cultures that were cotreated with PD98059 and ethanol exhibited markedly enhanced levels of cartilage matrix accumulation, relative to cultures incubated in the presence of the inhibitor alone.

Treatment of cultured limb mesenchyme cells with 1 μ M Wortmannin also resulted in a stimulation of chondrogenesis. Moreover, the presence of Wortmannin did not block the ability of ethanol to stimulate *in vitro* chondrogenesis. Although a potent and selective inhibitor of PI-3 kinase, Wortmannin also inhibits signaling through the ERK MAP kinase pathway at concentrations greater than 200 - 300 nM (Ferby et al., 1994; Powis et al., 1994; Duckworth and Cantley, 1997). Thus, it would appear that neither the ERK MAP kinase signaling pathway nor the PI-3 kinase signaling pathway is required for ethanol to exert its stimulatory effects on chondrocyte differentiation.

In contrast, SB202190, a potent inhibitor of p38 MAP kinase, but not of the ERK MAP kinase subgroup, did not itself exert a stimulatory effect on the differentiation of limb mesenchyme cells. These results suggest that the p38 MAP kinase pathway may not play a necessary role in spontaneous chondrogenesis. Moreover, when micromass cultures were cotreated with SB202190 and ethanol, chondrogenesis was markedly enhanced compared to cultures treated with SB202190 alone. This suggests that an active p38 MAP kinase pathway is also not required for ethanol to stimulate cartilage differentiation *in vitro*.

4.10.3 Protein Kinase A (PKA) Inhibitors

During the mesenchyme cell condensation phase of chondrogenic differentiation, endogenous cAMP levels increase dramatically (Solursh et al., 1981a; Rodgers et al., 1989). cAMP is a PKA agonist, and as such, it has been postulated that PKA signaling may play a regulatory role in chondrogenesis. Indeed, it has been shown that exogenous dibutyryl cAMP enhances chondrogenic differentiation in micromass cultures of limb mesenchyme through activation of PKA, and the subsequent phosphorylation of CREB (Lee and Chuong, 1997). In contrast, treatment of cultured limb mesenchyme cells with a PKA inhibitor, H-8, was reported to suppress spontaneous cartilage formation, and to block cAMP-induced cartilage differentiation (Lee and Chuong, 1997). However, the H-8 inhibitor employed in Lee and Chuong's study may not have exclusively blocked PKA signaling at the 20 μ M - 60 μ M doses used. These concentrations greatly exceed the K_i (1.2 μ M) of H-8 for selective PKA inhibition, and are levels that have been reported to non-specifically inhibit other cyclic nucleotide-dependent kinases as well. Moreover, in the study of Lee and Chuong (1997), the extent of chondrogenic differentiation as measured by Alcian blue-positive matrix accumulation was never normalized against total cell number or DNA content. Therefore, the reported suppressive effects of H-8 on chondrogenesis may have been a secondary consequence of reduced cell proliferation or viability, and it is reasonable to question the validity of their interpretation.

Indeed, in the present study, I observed no such suppression of chondrogenesis when limb mesenchyme cells were cultured in the presence of two other PKA inhibitors, H-89 and myristoylated PKA inhibitor amide. More importantly, limb mesenchyme cultures that were treated with ethanol in the presence of either H-89 or PKA inhibitor amide demonstrated a marked increase in Alcian blue-positive matrix deposition/ μ g DNA, relative to cultures that were incubated with each of the PKA

inhibitors alone. These results suggest that ethanol may not require the PKA pathway to stimulate cartilage differentiation in micromass culture.

4.10.4 Protein Tyrosine Kinase (PTK) Inhibitors

I also employed a number of pharmacological PTK inhibitors (Lavendustin A, Erbstatin, Tyrphostin, and Genistein) to block intracellular signaling through a variety of tyrosine kinases including the Epidermal Growth Factor Receptor (EGFR). When limb mesenchyme cells were cultured in the presence of both 1.5% ethanol and any one of these PTK inhibitors, cartilage differentiation was dramatically enhanced relative to cultures treated with each of the inhibitors alone. Thus, none of the tyrosine kinase inhibitors tested blocked the ability of ethanol to stimulate chondrogenesis. Surprisingly, none of the PTK inhibitor treatments alone caused any significant suppression of spontaneous chondrogenesis in micromass culture, despite the general importance of tyrosine kinases in cell differentiation and growth control. However, a tremendous diversity of tyrosine kinases have been implicated in cell signaling, and it is quite possible that many such kinases were unaffected by the specific PTK inhibitors used in this study.

Unfortunately, the various protein kinase inhibitor experiments conducted in the present study did not effectively clarify the role of signal transduction pathways in ethanol-induced chondrogenesis. Many of the pharmacological agents tested did not affect spontaneous chondrogenesis, and most did not prevent ethanol from exerting its stimulatory effects on cartilage differentiation. An exception was the PKC downregulators (PMA, Mezerein, and OIL) which dramatically suppressed spontaneous cartilage formation by limb mesenchyme cells *in vitro*, and greatly reduced the ability of ethanol to promote chondrogenesis. However, the contrasting lack of effect of PKC inhibitors (Bisindolylmaleimide, Chelerythrine, and H-7) on ethanol's stimulatory actions makes even the role of PKC unclear.

4.11 The Potential Relationship of Alcohol's Stimulatory Effects on *In Vitro* Cartilage Differentiation to its Teratogenic Effects on *In Vivo* Skeletogenesis

The present study demonstrates that ethanol potently stimulates chondrogenic differentiation in cultures of prechondrogenic mesenchyme isolated from the limb and facial primordia of the chicken embryo. An exciting and intriguing possibility is that ethanol's stimulatory effects on *in vitro* cartilage formation might bear a mechanistic

relationship to the well-documented teratogenic effects of ethanol on *in vivo* embryogenesis.

Ethanol's teratogenic effects have usually been attributed to disturbances of systemic physiological processes, such as oxygen uptake, placental transport, glucose utilization, vascular insufficiencies, cellular toxicity, etc. (Streissguth et al., 1980; Sulik et al., 1981; Hoyseth and Jones, 1989; Webster, 1989; Schenker et al., 1990; Seyoum and Persaud, 1990; Kotch and Sulik, 1992; Kotch et al., 1992; Zajac and Abel, 1992; Henderson et al., 1995; Shibley and Pennington, 1997). However, experimental investigations of ethanol's deleterious effects on embryogenesis have traditionally employed *in vivo* systems or whole embryo cultures, where it would be difficult to resolve systemic influences from more selective effects on individual target cell populations. The high incidence of facial dysmorphologies associated with gestational ethanol exposure in both humans and laboratory animals (Webster and Ritchie, 1991; Weston et al., 1994) suggest that neural crest cells of the embryonic facial primordia might indeed possess a relatively acute sensitivity to alcohol-mediated perturbations of growth and differentiation. Recent investigations support this conclusion, demonstrating that ethanol exposure preferentially depresses the viability and/or motility of cranial neural crest cells at the time they migrate from the converging neural folds into the developing facial primordia (Cartwright and Smith, 1995a,b; Rovasio and Battiato, 1995; Chen and Sulik, 1996). To our knowledge, my study is the first to demonstrate that ethanol might, in addition, influence the extent of chondrogenic differentiation by prechondrogenic limb and facial mesenchyme cells. We speculate that if ethanol can elevate chondrogenic differentiation *in vivo* as it does *in vitro*, then malformations might arise through the induction of precocious, excessive, prolonged, or ectopic cartilage formation within embryonic limb and facial primordia. The vertebrate facial skeleton, for example, develops in a complex fashion, through the formation of permanent and/or transient cartilaginous structures from the facial mesenchyme at some sites and through direct intramembranous ossification at others. Even slight perturbations in the relative extents of chondrogenic versus osteogenic differentiation in the developing facial primordia might conceivably lead to the types of subtle facial dysmorphologies that are most commonly associated with prenatal alcohol exposure. An enhancement of chondrogenesis in the developing limb might potentially account for the occurrence of certain limb malformations (e.g., syndactyly and polydactyly) following prenatal exposure to alcohol. Other limb abnormalities, such as the absence of digits or loss of more proximal limb structures, are more difficult to explain. However, it seems reasonable to suggest that these types of malformations may arise via

precocious induction of cartilage formation *in vivo*. Indeed, similar limb defects have been observed upon AER removal, which causes a precocious differentiation of cells within the underlying progress zone and a cessation of P-D limb outgrowth (Saunders et al., 1957).

It is not yet apparent whether there is any direct relationship between ethanol's stimulatory effects on *in vitro* chondrogenesis and its teratogenic effects on skeletogenesis *in vivo*. However, it appears that there are certain similar properties between the two. In my study, maximal stimulation of chondrogenesis in cultures of embryonic limb and facial mesenchyme occurred at concentrations of 1.5 - 2.0% ethanol and 1.0 - 1.5% ethanol, respectively (approximately 175 - 350 mM), concentrations that would approach lethality in most animals and humans *in vivo*. However, a statistically significant increase in cartilage matrix GAG accumulation in both limb and facial mesenchyme cultures was observed with as little as 0.5% alcohol (85 mM). Blood alcohol levels approximating the latter can be attained in humans after excessive drinking and are tolerated by alcoholics (Lindblad and Olsson, 1976; Davis and Lipson, 1986). Indeed, even in genetically-sensitive strains of mice, alcohol-related anatomical defects can be induced consistently only after high, near-lethal levels of gestational ethanol exposure (approximately 85-175 mM) (Sulik et al., 1981; Webster et al., 1983; Webster, 1989; Webster and Ritchie, 1991). Therefore, the range of ethanol doses which induces significant *in vitro* effects on chondrogenesis overlaps the dose range for *in vivo* alcohol teratogenicity. Within micromass cultures, high levels of alcohol had to be sustained throughout the culture period in order for ethanol to exert a stimulatory effect on cartilage matrix formation. Limb mesenchyme cells that were transiently exposed to 1.5 - 2.0% ethanol for the first day or two of culture, and then incubated in untreated control medium for the remainder of the 3-day culture period did not elaborate significantly greater Alcian blue-positive cartilage matrix than parallel controls (preliminary data; not shown). Similarly, acute levels of alcohol appear to be required for a relatively prolonged period *in vivo* to induce fetal alcohol-related birth defects in humans. Indeed, there have been few, if any, instances of a child with FAS/FAE being born to a woman who was not a chronic alcoholic (Abel, 1999).

4.12 Future Directions

There are numerous questions that remain unanswered, and many intriguing avenues for future investigation. For example, my findings suggest that ethanol may stimulate cartilage differentiation *in vitro* by modulating the response of prechondrogenic mesenchyme cells to one or more specific serum factors. Indeed, I

have demonstrated that ethanol treatment rapidly downregulates endogenous TGF- β_3 mRNA expression in micromass cultures of limb mesenchyme, and upregulates expression of transcripts for BMPR-IB. Therefore, it would be worthwhile to determine whether ethanol exposure influences the expression and activity of receptors for a variety of serum factors (e.g., FGFs, IGFs, TGF- β s and BMPs) that have been implicated at some level in the regulation of cartilage formation. This would involve obtaining or generating specific probes for use in RNA dot-blot or Northern analysis, as well as antibodies for immunocytochemical detection of their protein product. Since, in other cell types, ethanol exposure can modulate interactions between signaling ligands and their receptors (Henderson et al., 1989; Resnicoff et al., 1993; Tomono and Kiss, 1995), it would be valuable to examine whether ethanol can stimulate chondrogenesis following functional inactivation of signaling through FGF, IGF, TGF- β and BMP intracellular pathways. This could be accomplished using retrovirally-expressed dominant-negative receptor constructs, as in the recent study of BMP receptor signaling performed by Zou et al. (1997). These studies should clarify not only the specific molecular pathways through which ethanol stimulates chondrocyte differentiation, but also the extent to which activation of these growth factor pathways is required for the initiation of chondrogenesis.

Serum also contains a number of other soluble factors that have been implicated as regulators of chondrogenic differentiation. For example, elevated prostaglandin production is associated with mesenchyme cell condensation (Chepenik et al., 1984; Biddulph et al., 1988; Capehart and Biddulph, 1991). One pathway through which ethanol has been found to influence cellular metabolism in a number of cultured cell types is through perturbation of cellular prostaglandin production (Szabo et al., 1992; Balsinde, 1993; Navamani et al., 1997). Indeed, it has been suggested that interference with embryonic and/or placental prostaglandin metabolism might be a significant source of alcohol-related birth defects (Anggard, 1983; Randall et al., 1987). Accordingly, it seems logical to postulate that ethanol treatment might stimulate cartilage differentiation *in vitro* by influencing the level of prostaglandin synthesis in prechondrogenic mesenchyme cells. Initial experiments could examine whether ethanol treatment elevates endogenous prostaglandin production in cultures of embryonic chick limb mesenchyme. One could also examine whether ethanol exposure potentiates the response of prechondrogenic limb mesenchyme cells to exogenous prostaglandins. It has been demonstrated that prostaglandins influence chondrogenic differentiation through the activation of the intracellular cAMP second messenger system (Gay and Kosher, 1984; Biddulph et al., 1988). If ethanol enhances chondrogenesis in cultured

limb mesenchyme cells by potentiating cellular responses to prostaglandin stimulation, one would anticipate a greater prostaglandin-induced increase in cAMP levels in alcohol-treated mesenchymal cells than in control cells. Subsequent experiments could then be performed to determine whether inhibitors of prostaglandin synthesis can block the stimulatory influence of ethanol on *in vitro* chondrogenesis. Prostaglandin and cAMP activity would be assayed using commercially available kits.

The fact that in virtually all cases examined, ethanol significantly stimulated chondrogenesis following presumed inactivation of a particular kinase pathway suggests that none of these individual pathways is absolutely required for ethanol to exert its effects. The possibility remains, however, that ethanol may stimulate cartilage differentiation via simultaneous effects on more than one of these pathways. Thus, inactivation of a single pathway may not be sufficient to block the stimulatory effects of ethanol. This possibility could be experimentally addressed by simultaneously blocking more than one signal transduction pathway, either with the use of more than one inhibitor, or with dominant-negative forms of these protein kinases. Alternatively, ethanol may enhance chondrogenic differentiation by activating some other intracellular kinase signaling pathway (e.g., Ca^{2+} /calmodulin-dependent kinase, casein kinase, glycogen synthase kinase, etc.) that was not examined in this study. As such, I believe that a more thorough examination of the role of specific intracellular signal transduction pathways in mediating ethanol's effects on chondrogenesis is warranted. In addition to using pharmacological inhibitors or dominant-negative constructs to block signaling through various intracellular signaling pathways, it would be ideal to directly monitor the effects of ethanol on the activity of specific protein kinases in cultured prechondrogenic mesenchyme cells.

The possibility also exists that ethanol might stimulate chondrogenesis by inhibiting some specific intracellular signaling pathway that negatively regulates chondrogenesis. Indeed, the ability of staurosporine, a broad-specificity kinase inhibitor, to enhance *in vitro* chondrogenesis suggests the existence of kinase pathways that act as negative regulators of chondrocyte differentiation (Kulyk, 1991). The ability of PD98059 and Wortmannin to enhance chondrogenesis in micromass culture is also consistent with this possibility. This possibility could be addressed by examining the effects of ethanol treatment on specific downstream targets, if known, of particular protein kinases.

An intriguing question is whether there is a mechanistic relationship between the teratogenic effects of retinoids and alcohols on limb and facial skeletogenesis. As suggested by the present study, it is unlikely that ethanol stimulates chondrogenesis by

competing with retinol for ADH. However, this doesn't discount the possibility that ethanol may interfere with retinoid signaling via some other mechanism. Therefore, it would be of importance to examine whether ethanol exposure influences the expression of transcripts and/or encoded products of various intracellular retinoic acid/retinoid receptors (RARs and RXRs) in micromass cultures of limb mesenchyme. This could be addressed using Northern RNA analysis, and either Western blot analysis or immunocytochemistry, respectively. Other experiments could examine whether ethanol modulates the functional activity of these receptors. One might attempt to block RA signaling by ectopically expressing dominant-negative RARs and RXRs in limb mesenchyme cell cultures, or through the use of specific pharmacological inhibitors.

In the present study, I have demonstrated the effects of ethanol treatment on the expression of gene transcripts for several putative chondrogenic regulatory genes. An important question, however, is whether ethanol may be acting to recruit undifferentiated mesenchyme cells into the chondrocyte differentiation pathway or whether it is merely enhancing the expression of cartilage-specific genes in cells that are already committed to differentiate. Whole mount in situ hybridization methods would be very useful in addressing this question. It also remains to be determined whether ethanol treatment influences the level of expression of the encoded protein products of these genes. An investigation of *Sox-9* at this level seems particularly crucial, given its role as a transcription factor that regulates the expression of the type II collagen gene. Western blot analysis could be used to examine whether observed increases in *Sox-9* mRNA expression are paralleled by an increase in the levels of *Sox-9* protein. Gel mobility shift analysis could also be conducted to determine whether Col2A1 promoter-binding activity of the *Sox-9* transcription factor is modulated in response to ethanol. Although it appears that ethanol acts upstream of Col2A1 in enhancing *Sox-9* mRNA expression in cultured limb mesenchyme, it is unclear as to whether ethanol may be acting directly on *Sox-9* or further upstream in the chondrocyte differentiation pathway. Accordingly, it would be worthwhile to analyze the *Sox-9* promoter region, using DNA footprinting analysis, to identify sequences that are bound by nuclear proteins that might be activated in response to ethanol exposure. It would also be of interest to examine the effects of ethanol treatment on the expression of transcripts for other regulatory genes and signaling molecules that have been implicated as regulators of skeletal pattern formation in the developing limb (e.g., *Shh*, *Wnt-7A*, *Hox D*, etc.).

In the present study, I attempted to identify novel genes that are preferentially expressed during ethanol-induced chondrocyte differentiation, using "Differential

Display" PCR cloning. Although I was forced to discontinue this line of experiments due to technical difficulties and time constraints, I still believe that this remains a worthy objective. An important advantage to using such a technique is that one does not have to make any prior assumptions as to the specific nature of the intracellular pathways through which ethanol may be exerting its stimulatory effects on cartilage differentiation. A possible alternative to the differential display method is the "suppressive PCR" technique (e.g., Clontech PCR-Select cDNA Subtractive Hybridization).

As yet, there is no direct evidence that alcohol's stimulatory effects on *in vitro* cartilage differentiation are mechanistically-related to the teratogenic effects of alcohol on *in vivo* skeletogenesis. A demonstration that alcohol can enhance chondrogenesis by mesenchymal cells of rat or murine limb and facial primordia, as it does in corresponding cells of the chick embryo, would be important toward establishing the potential relevance of my *in vitro* studies to ethanol teratogenicity in humans. Indeed, it is certainly possible that there may be differences in the ethanol-responsiveness of tissues from different organisms. Therefore, a simple, yet important question to be addressed is whether ethanol treatment stimulates cartilage differentiation in cultures of prechondrogenic mesenchyme isolated from the limbs and facial primordia of rat or murine embryos.

Another important question to address is whether ethanol exposure upregulates or precociously activates cartilage-specific gene expression *in vivo* (e.g., type II collagen, aggrecan, *Sox-9*) as it does *in vitro*. This could involve exposing chick or mouse embryos to teratogenic doses of ethanol during critical stages of development, and then carrying out *in situ* hybridization analyses with gene-specific probes. Analysis of ethanol's effect on embryonic chondroprogenitor cells *in vivo*, however, is complicated by the fact that ethanol exerts a multitude of nonspecific, systemic effects on embryogenesis. Therefore, it would also be worthwhile to examine the effects of ethanol treatment on organ cultures of whole limb buds. This *in vitro* system would approximate the *in vivo* setting in that local tissue interactions and intercellular communications in the developing limb are maintained.

I have demonstrated that ethanol exposure induces substantial cartilage formation by maxillary mesenchyme cells which are not spontaneously chondrogenic *in vitro*, and which form bone rather than cartilage *in vivo*. As such, it would be interesting to examine whether ethanol promotes chondrogenic differentiation in facial mesenchyme cells at the expense of suppressing the alternate, osteogenic pathway of differentiation. To address this possibility, the expression of several osteogenic

differentiation markers (e.g., alkaline phosphatase, bone sialoprotein, osteonectin, etc.) could be examined in micromass cultures of chick embryonic facial mesenchyme in response to ethanol.

Collectively, such studies would yield greater insight into the molecular mechanisms through which alcohol affects chondrocyte differentiation *in vitro*, and might eventually help to elucidate the mechanisms responsible for the teratogenic effects of alcohol on embryonic skeletal development *in vivo*.

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